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LIPOLYTIC ENZYME VARIANTS HAVING PHOSPHOLIPASE ACTIVITY

FIELD OF THE INVENTION

The present invention relates to a variant of a parent lipolytic enzyme, and more specifically to such a variant having higher phospholipase activity than the parent lipolytic enzyme. The invention also relates to a DNA sequence encoding the variant, a vector comprising the DNA sequence, a transformed host cell harboring the DNA sequence or the vector, to a method of producing the variant, and to methods of using the variant.

BACKGROUND OF THE INVENTION

Phospholipases are useful in a variety of industrial applications, e.g. in baking and treatment of vegetable oil to reduce the content of phospholipid.

Phospholipases are known from a number of biological sources, including animal sources and microorganisms.

F. Hara et al., JAOCS, 74 (9), 1129-32 (1997) indicates that some lipases have a certain phospholipase activity, whereas most lipases have little or no activity on phospholipids. Thus, phospholipase activity has been described in the lipases from guinea pig pancreas, *Fusarium oxysporum* and *Staphylococcus hyicus*, and attempts have been made to relate the phospholipase activity to the structure of the lipase. WO 98/26057; M.D. van Kampen et al., Chemistry and Physics of Lipids, 93 (1998), 39-45; A. Hjorth et al., Biochemistry 1993, 32, 4702-4707.

SUMMARY OF THE INVENTION

The inventors have found that starting from a given parent lipolytic enzyme, it is possible to make variants with a higher phospholipase activity by modifying the amino acid sequence in the vicinity of the C-terminal and the alcohol binding site.

Accordingly, the invention provides a variant of a parent lipolytic enzyme, which variant:

a) comprises an alteration which is an insertion, a deletion or a substitution of an amino acid residue, and

b) has a higher phospholipase activity than the parent lipolytic enzyme.

The alteration may be at a position which is within 10 amino acid positions from the C-terminal of the mature protein or corresponds to such a position in the *H. lanuginosa* lipase. The parent lipolytic enzyme may particularly be one having a lid and an alcohol binding site, and the alteration may alternatively be at a position which is

i) no more than 10 Å from the C atom at the sn2 position of the glycerol part of a substrate triglyceride, or

ii) in the lipolytic enzyme lid.

The invention also provides a DNA sequence encoding the variant, an expression vector comprising the DNA sequence, a transformed host cell harboring the DNA sequence or the expression vector, and to a method of producing the variant by cultivating the transformed host cell so as to produce the variant and recovering the variant from the resulting broth. Further, the invention provides processes using the variant in baking or degumming of vegetable oil.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows an alignment of lipase sequences.

Figs. 2-3 show firmness and elasticity, respectively, of bread. Details are described in the Examples.

DETAILED DESCRIPTION OF THE INVENTION

Parent lipolytic Enzyme

The lipolytic enzyme to be used in the present invention is one that can hydrolyze ester bonds. Such enzymes include, for example, lipases, such as triacylglycerol lipase (EC 3.1.1.3), lipoprotein lipase (EC 3.1.1.34), monoglyceride lipase (EC 3.1.1.23), lysophospholipase, ferulic acid esterase and esterase (EC 3.1.1.1, EC 3.1.1.2). The numbers in parentheses are the systematic numbers assigned by the Enzyme Commission of the International Union of Biochemistry in accordance with the type of the enzymatic reactivity of the enzyme.

The parent lipolytic enzyme may have a lid and an alcohol binding site, e.g. a fungal lipolytic enzyme. Examples of such parent lipolytic enzymes are lipolytic enzymes of the *Humicola* family and the *Zygomycetes* family.

The *Humicola* family of lipolytic enzymes consists of the lipase from *H. lanuginosa* strain DSM 4109 and lipases having more than 50 % homology with said lipase. The lipase from *H. lanuginosa* (synonym *Thermomyces lanuginosus*) is also referred to as Lipolase®. It is described in EP 258 068 and EP 305 216, and has the amino acid sequence shown in positions 1-269 of SEQ ID NO: 2 of US 5,869,438.

The *Humicola* family also includes the following lipolytic enzymes: lipase from *Penicillium camembertii* (E04336), lipase/phospholipase from *Fusarium oxysporum* (EP 130064, WO 98/26057), lipase from *F. heterosporum* (R87979), lysophospholipase from *Aspergillus foetidus* (W33009), lipase from *A. oryzae* PLA1 (W58541), lipase from

A. oryzae (D85895), lipase/ferulic acid esterase from *A. niger* (Y09330), lipase/ferulic acid esterase from *A. tubingensis* (Y09331), lipase from *A. tubingensis* (WO 98/45453), lysophospholipase from *A. niger* (WO 98/31790).

The *Zygomycetes* family comprises lipases having at least 50 % homology with the lipase of *Rhizomucor miehei* (P19515). This family also includes the lipases from *Absidia reflexa*, *A. sporophora*, *A. corymbifera*, *A. blakesleeana*, *A. griseola* (all described in WO 96/13578 and WO 97/27276) and *Rhizopus oryzae* (previously *R. niveus*, *R. delemar*, *R. japonicus*, P21811). Numbers in parentheses indicate publication or accession to the EMBL, GenBank, GeneSeqp or Swiss-Prot databases.

It is of particular interest to derive a variant with phospholipase activity from a parent lipolytic enzyme having no or very little phospholipase activity, e.g. corresponding to a ratio of phospholipase activity to lipase activity below 0.1 PHLU/LU or below 50 PHLU/mg.

Lipolytic enzyme variants

The lipolytic enzyme variant of the invention comprises an alteration (i.e., an insertion, a deletion or a substitution of an amino acid residue), at a position in one of the following overlapping regions:

i) near the C-terminal, i.e. within 10 amino acid positions from the C-terminal or corresponding to such a position in the *H. lanuginosa* lipase, or

ii) at the "alcohol binding site", i.e. no more than 10 Å from the C atom at the sn2 position of the glycerol part of a substrate triglyceride, or

iii) in the lid.

The total number of alterations in the above regions is typically not more than 20, e.g. not more than 10 or not more than 5, and there may be as little as 1 or 2 alterations in the above regions.

The variant of the invention may further comprise a peptide extension at the N-terminal. This peptide extension preferably consists of 1-15 (particularly 4-10) amino acid residues, and preferably comprises 1, 2 or 3 positively charged amino acids.

Some preferred peptide extensions are AS, SPIRR, E1RP, E1SPIRPRP, E1SPPRRP and E1SPIRPRP. Further, any peptide extension described in WO 97/04079 and WO 97/07202 may be used.

In addition, the lipolytic enzyme variant of the invention may optionally include other modifications of the parent enzyme, typically not more than 10, e.g. not more than 5 alterations outside of th above regions.

Alterations near C-terminal

As stated above, the variant of the invention may have one or more alterations within 10 amino acid positions from the C-terminal of the mature protein, or at positions corresponding to such positions in the *H. lanuginosa* lipase, i.e. positions 260-269 of the *H. lanuginosa* lipase. Corresponding positions may be found by alignment of the two sequences as described later in this specification.

The lipolytic enzyme variant may be truncated by deleting amino acid residues corresponding to the first 1, 2, 3, 4, 5 or 6 positions at the C-terminal. A truncated variant may have improved thermostability.

Alternatively, the variant may carry a peptide extension at the C-terminal and/or the N-terminal. The C-terminal extension may consist of 1-10 amino acid residues, e.g. A, P, AG, DG, PG, AGG, PVGF, AGRF, PRGF, AGGF or AGGFS; or it may consist of 40-50 residues, e.g., consisting of the 48 C-terminal residues of the *Fusarium oxysporum* lipase AGGFSWRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS. The C-terminal extension may increase the phospholipase activity.

Some alterations in the region overlapping with the alcohol binding site are described below.

A preferred alteration is a substitution at a position corresponding to G266 in the *Humicola lanuginosa* lipase, preferably with an amino acid of intermediate size, e.g. A, C, D, N, L, I, S, T, P or V. Such alteration alone has been found sufficient to increase the phospholipase activity.

Other preferred alterations are such that alter the tertiary structure, e.g. by introducing bulky side chains or by disrupting the bond angles, e.g. by introducing Pro. Such alterations may be made at positions corresponding to positions G263, L264, I265, T267 or L269 in the *Humicola lanuginosa* lipase. Some preferred substitutions are G263A,E,Q,R; L264A,C,P,Q; I265L,N,T; T267A,Q or L269N.

Alteration in lipase lid

As stated above, the amino acid sequence of the parent lipolytic enzyme may be modified in the lid region of the lipase described in Brady et al., Nature 343, 1990, pp. 767-770 and in Brzowski A M et al., Nature, 351: 491 (1991). In the *H. lanuginosa* lipase, the lid is located at positions 80-100, and the modification may particularly be made at positions 82-98.

The variant typically contains no more than 5 alterations in the lid region; it may contain 0, 1, 2 or 3 alterations. A preferred alteration is a substitution of an amino acid corresponding to G91, D96 and/or E99 in the *Humicola lanuginosa* lipase with a neutral or positiv ly charged amino acid, e.g. a substitution corresponding to G91A, D96S,W and/or E99K.

Alt ration in alcoh l binding site

As already stated, the amino acid sequence of the parent lipolytic enzyme may be modified at a position which is within 10 Å (e.g. within 8 Å, particularly within 6 Å) of the C atom at the sn2 position of the glycerol part of a substrate triglyceride. This region will be referred to as the "alcohol binding site" of the lipase; it is described in Brzowski A M et al., Nature, 351: 491 (1991); Uppenberg et al., Biochemistry, 1995, 34, 16838-16851; A. Svendsen, Inform, 5(5), 619-623 (1994).

The variant typically contains no more than 10 alterations in the alcohol binding site, e.g. 1, 2, 3, 4, 5 or 6 alterations.

The alteration may particularly be in that part of the alcohol binding site which comes within 20 positions (e.g. within 10 positions) of the C-terminal.

For the *Rhizomucor miehei* lipase, the extent of the alcohol binding site can be found from the PDB file "5tgl.pdb" available in Structural Classification of Proteins (SCOP) on the Internet, at http://pdb.pdb.bnl.gov, showing the complex with the inhibitor n-hexylphosphonate ethyl ester which mimics the substrate. This is described in Brzowski et al. (supra) and Brady et al. (supra). The following amino acid positions lie within 10 Å of the sn2 position in the *Rhizomucor miehei* lipase: 25, 28, 80-84, 88, 143-146, 175, 203, 205, 254-255, 257-259, 264-267. The following are within 8 Å: 81-83, 144, 257-258, 265-267, and the following within 6 Å: 82, 144, 257, 266.

In the *Humicola lanuginosa* lipase, the following positions are within 10 Å of the sn2 position: 18, 21, 81-85, 89, 145-148, 172, 201, 203, 255-256, 258-260, 264-267. The following are within 8 Å: 82-84, 89, 146, 258-259, 265-267, and the following within 6 Å: 83, 146, 258, 266.

Homology and alignment

For purposes of the present invention, the degree of homology may be suitably determined by means of computer programs known in the art, such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-45), using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

In the present invention, corresponding (or homologous) positions in the lipase sequences of *Rhizomucor miehei* (rhimi), *Rhizopus delemar* (rhidl), *Thermomyces lanuginosa* (former; *Humicola lanuginosa*) (SP400), *Penicillium camembertii* (Pcl) and *Fusarium oxysporum* (FoLnp11), are defined by the alignment shown in Figure 1.

To find the homologous positions in lipase sequences not shown in the alignment, the sequence of interest is aligned to the sequences shown in Figure 1. The new

sequence is aligned to the present alignment in Fig. 1 by using the GAP alignment to the most homologous sequence found by the GAP program. GAP is provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-45). The following settings are used for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

Preferred variants

Some preferred variants of the *H. lanuginosa* lipase have the following modifications. Alterations given in parentheses are optional. Corresponding alterations may be made in other parent lipolytic enzymes.

(E1A)+ G91A+ D96W+ E99K+ P256A+ W260H+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S)

(E1A)+ G91A+ D96W+ E99K+ (E239C)+ Q249R+ P256A+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G +273F (+274S)

(E1A)+ G91A+ D96W+ E99K+ N248T+ Q249R+ W260Q+ G263Q+ L264A+ I265T+ G266DT267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S)

SPIRR+ G91A+ D96W+ E99K+ W260C+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272+ G273F (+274S)

SPIRR+ G91A+ D96W+ E99K+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S)

(E1A)+ G91A+ D96W+ E99K+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S)

(E1A)+ G91A+ D96W+ P256A+ W260H+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S)

SPIRR+ D96W+ E99K+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S)

SPIRR+ G91A+ D96W+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S)

(E1A)+ G91A+ D96W+ E99K+ P256A+ W260H+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N

(E1A)+ G91A+ D96W+ E99K+ Q249R+ G263E+ G266D+ L269N+ 270P+ 271V+ 272G+ 273F

(E1A)+ G91A+ D96W+ E99K+ Q249R+ G263A+ G266S+ L269N+ 270A+ 271G+ 272R+ 273F

(E1A)+ G91A+ D96W+ E99K+ Q249R+ L264P+ Δ266+ L269I+ 270P+ 271R+ 272G+ 273F

(E1A)+ G91A+ D96W+ E99K+ Q249R+ L264C+ I265N+ G266P+ T267stop
(E1A)+ G91A+ D96W+ E99K (+R232L)+ Q249R+ G266S+ 270A
(E1A)+ G91A+ D96W+ E99K+ Q249R+ G266S+ 270D+ 271G
(E1A)+ G91A+ D96W+ E99K+ Q249R+ L264F+ Δ266+ 270A+ 271G+ 272G+ 273F
(E1A)+ G91A+ D96W+ E99K+ Q249R+ L264G+ I265G+ G266F+ T267stop
(E1A)+ G91A+ D96W+ E99K+ Q249R+ L264stop
(E1A)+ G91A+ D96W+ E99K+ P256A+ W260H+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G
(E1A)+ G91A+ D96W+ E99K+ P256A+ W260H+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G
(E1A)+ G91A+ D96W+ E99K+ Q249R+ G266D
(E1A)+ G91A+ D96W+ E99K+ Q249R+ G266D
(E1A)+ G91A+ D96W+ E99K+ Q249R+ G266A+ 270P+ 271G
(E1A)+ G91A+ D96W+ E99K+ Q249R+ L264P+ I265F+ L269stop
(E1A)+ G91A+ D96W+ E99K+ Q249R+ G266D+ L269S+ 270A+ 271G+ 272G+ 273F
(E1A)+ G91A+ D96W+ E99K+ Q249R+ G266D+ L269N+ 270A
(E1A)+ G91A+ D96W+ E99K+ Q249R+ G266S+ L269N+ 270A+ 271G+ 272G+ 273F
(E1A)+ G91A+ D96W+ E99K+ Q249R+ L264P+ L267Q+ L269N
(E1A)+ G91A+ D96W+ E99K+ Q249R+ G263R+ I265L+ L269N+ 270P
(E1A)+ D96W+ E99K+ P256A+ W260H+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S)
(E1A)+ (G225R)+ G266D
(E1A)+ (G225R)+ G263A+ I265V+ G266S
(E1A)+ (G225R)+ G263A+ T267A
E1SPPCGRRP+ D96S+ E239C+ Q249R+ I252M+ L264Q+ G266D
E1SPPCGRRP+ G91A+ D96W+ E239C+ Q249R+ G266D
E1SPPCGRRP+ D96S+ E239C+ Q249R+ G266D
E1SPPCGRRP+ D96S+ E239C+ Q249R+ G266C+ L267A
(E1A)+ G91A+ D96W+ E99K+ Q249R+ G266A
(E1A)+ D96M+ (G106S)+ (G225R)+ G266D
(E1A)+ D96Q+ (G106S)+ (G225R)+ G266S
(E1A)+ D96F+ (G225R)+ G266S
(E1A)+ D96C+ (G225R)+ G266T
(E1A)+ D96H+ (G106S)+ (G225R)+ G266S
SPIRR+ D96S+ G266D

SPIRR+ D96R+ (G106S)+ G266D
SPIRR+ D96I+ (G106S)+ G266S
SPIRR+ D96W+ (K237R)+ G266S
SPIRR+ G266A
SPIRR+ D96S+ (G106S)+ (G225R)+ G266D
SPIRR+ D96Q+ (G106S)+ (G225R)+ G266A
SPIRR+ D96Y+ (G106S)+ (G225R)+ G266N
SPIRR+ D96C+ (G106S)+ (G225R)+ G266T
SPIRR+ D96H+ (T186I)+ (G225R)+ G266S
E1SPPRRP+ G91A+ D96W+ (E239C)+ Q249R+ G266D
E1SPPRRP+ G91A+ D96W+ (E239C)+ Q249R+ G266S
E1SPPRRP+ G91A+ D96W+ (E239C)+ Q249R+ G263E+ G266S+ 270A
E1SPPRRP+ G91A+ D96W+ (E239C)+ Q249R+ L264P+ G266S
E1SPPRRP+ G91A+ D96W+ (E239C)+ Q249R+ P256T+ G266D
E1SPPRRP+ G91A+ D96W+ (E239C)+ Q249R+ G266C+ T267P+ L269stop
(E1A)+ G91A+ D96W+ E99K+ Q249R+ G266S+ T267S
E1SPPCGRRP+ G91A+ D96W+ E239C+ Q249R+ P256T+ G266S
E1SPPCGRRP+ E239C+ Q249R+ P256T+ G266S+ T267A
E1SPPCGRRP+ E239C+ Q249R+ G266D
E1SPPCGRRP+ G91A+ D96W+ E239C+ Q249R+ G266D
E1SPPRRP+ D96S+ (E239C)+ Q249R+ G266D
G266D
E1SPPCGRRP +E99N +E239C +Q249R +G266D
E1SPPCGRRP +E239C +Q249R +G266D
E1SPPCGRRP +L93K +E99K +E239C +Q249R +G266D
E1SPPCGRRP +E99K +E239C +Q249R +G266D
G266A
G266W
G266E
G266V
G263Q +L264A +I265T +G266D +T267A
G263F +L264A +G266S +T267E
E1SPPCGRRP +E239C +Q249R +G263Q +L264A +I265T +G266D +T267A
G266S
G266K
G266L
G266R
G263A +G266A

G263A +G266Y
E1SPPCGRRP +E239C +Q249R +G266A
E1SPPCGRRP +E239C +Q249R +G266S
E1SPPCGRRP +E239C +Q249R +G263F +L264A +G266S +T267E
D62A + G266A
D62A + G266S
D96S + G266A
D96S+ G266S
D96S+ G266R
D96S+ G266W
D96S+ G266V
E1SPPCGRRP + G91A+ D96W+ (E239C)+ Q249R+ G266D
E1SPPCGRRP + G91A+ D96W+ (E239C)+ Q249R+ G266S
E1SPPCGRRP + G91A+ D96W+ (E239C)+ Q249R+ G263E+ G266S+ 270A
E1SPPCGRRP + G91A+ D96W+ (E239C)+ Q249R+ L264P+ G266S
E1SPPCGRRP + G91A+ D96W+ (E239C)+ Q249R+ P256T+ G266D
E1SPPCGRRP + G91A+ D96W+ (E239C)+ Q249R+ G266C+ T267P+ L269stop
G263D +L264I +I265N +G266E +T267GS
L264I +I265N +G266T +T267GL
E219G +L264I +I265N +G266T +T267GL
E1SPPCGRRP +D96S +E239C +Q249R +G263D +L264I +I265N +G266E +T267GS
E1SPPCGRRP +D96S +E239C +Q249R +L264I +I265N +G266T +T267GL
D96F +G266A
D96F +G266S
E1SPPCGRRP +E99N +E239C +Q249R +G266A
E1SPPCGRRP + D96S +E239C +Q249R +G266A
E1SPPCGRRP + D96S +E239C +Q249R +G266S

The above variants with (274S) in parentheses may optionally have a further C-terminal extension of WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNN-QARS (corresponding to the C-terminal of the *F. oxysporum* lipase) in full or truncated form.

Nomenclature for amino acid alterations

The nomenclature used herein for defining mutations is basically as described in WO 92/05249. Thus, G91A indicates substitution of G in position 91 with A. T267A,Q indicates substitution of T at position 267 with A or Q. T267stop indicates a stop codon,

i.e. deletion of T267 and all following amino acids (i.e. C268 and L269). 270P, 271V indicates a C-terminal extension of PV (i.e. at new positions 270 and 271). -G266 indicates deletion of G at position 266. Parentheses indicate that the alteration is optional, or in examples that the alteration is uncertain. SPIRR indicates an N-terminal extension. D266 may refer to the position or to substitution with any amino acid (except D).

E1SPPCGRRP indicates a substitution of E1 with SPPCGRRP, i.e. a peptide addition at the N-terminal. T267GS indicates a substitution of T267 with GS, or in other words the substitution T267G and an insertion of S between G267 and C268.

Phospholipase activity

The variant of the invention has higher phospholipase activity than the parent lipolytic enzyme. By the monolayer method described later in this specification, the variant preferably has a phospholipase activity of at least 0.1 nmol/min at pH 5.

By the PHLU method described later in this specification, the variant preferably has a phospholipase activity of at least 100 PHLU/mg (mg of pure enzyme protein), particularly at least 500 PHLU/mg. The variant has a ratio of phospholipase activity to lipase activity (both measured at pH 7) of at least 0.1 PHLU/LU, preferably at least 0.5, particularly at least 2.

The variants of the invention have the ability to hydrolyze intact phospholipid, as demonstrated by the PHLU method. They may have A_1 and/or A_2 activity, so they may be able to hydrolyze one or both fatty acyl groups in the phospholipid.

pH optimum

As illustrated in the Examples, some variants of the invention have an alkaline pH optimum for lipase activity and an acid pH optimum for phospholipase activity (e.g. pH 9-10 for lipase and pH 4-6 for phospholipase). Such variants can be used at acid pH (e.g. in baking or oil degumming, described later), as phospholipases with very low concomitant lipase activity.

Thermostability

The thermostability of the variant can conveniently be evaluated by means of Differential Scanning Calorimetry (DSC). Depending on exact mutations, the variants of the invention generally have similar or slightly lower thermostability than the parent lipolytic enzyme.

The temperature at the top of the denaturation peak (T_d) of the lipase from *Humicola lanuginosa* when heated at 90 deg/hr at pH 5 is just above 70 °C (=T_d). T_d for the variants of the invention is generally 5-10 degrees lower

Use of variant

The variant of th invention may be used for any purpose where phospholipase activity is desired. Depending on the intended use, the lipase activity can be increased or suppressed by a suitable choice of variant and of pH. Thus, the variant can be used in baking and in detergents, as described below.

The variant of the invention can also be used to improve the filterability of an aqueous solution or slurry of carbohydrate origin by treating it with the variant. This is particularly applicable to a solution or slurry containing a starch hydrolysate, especially a wheat starch hydrolysate since this tends to be difficult to filter and to give cloudy filtrates. The treatment can be done in analogy with EP 219,269 (CPC International).

The variant of the invention can be used in a process for reducing the content of phospholipid in an edible oil, comprising treating the oil with the variant so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil. This process is applicable to the purification of any edible oil which contains phospholipid, e.g. vegetable oil such as soy bean oil, rape seed oil and sunflower oil. The treatment is preferably carried out at acid pH, e.g. pH 3-5. Advantageously, at acid pH the variant of the invention has a high phospholipase activity and a low lipase activity, due to different pH optima of the two activities.

The process for oil treatment can be conducted according to principles known in the art, e.g. in analogy with US 5,264,367 (Metallgesellschaft, Röhm); K. Dahlke & H. Buchold, INFORM, 6 (12), 1284-91 (1995); H. Buchold, Fat Sci. Technol., 95 (8), 300-304 (1993); JP-A 2-153997 (Showa Sangyo); or EP 654,527 (Metallgesellschaft, Röhm).

The variant of the invention can further be incorporated in a detergent or rinse composition or in leather treatment, as described in JP-A 7-177884 (Kao). The variant may also be used in the processing of dairy and other food products, e.g. as described in EP 567,662 (Nestlé), EP 426,211 (Unilever), EP 166,284 (Nestlé), JP-A 57-189638 (Yakult) or US 4,119,564 (Unilever).

The variant can be used to prepare lyso-phospholipid (e.g. lyso-lecithin) by treating the corresponding phospholipid with the variant, e.g. as described in EP 870840, JP-A 10-42884, JP-A 4-135456 or JP-A 2-49593. The variant can also be used to make mayonnaise, e.g. as described in EP 628256, EP 398666 or EP 319064.

Use of variant in baking

The variant of the invention can be used in the preparation of dough, bread and cakes, e.g. to increase dough stability and dough handling properties, or to improve the elasticity of the bread or cake. Thus, the variant can b used in a process for making bread, comprising adding the variant to the ingredients of a dough, kneading the dough

and baking the dough to make the bread. This can be done in analogy with US 4,567,046 (Kyowa Hakko), JP-A 60-78529 (QP Corp.), JP-A 62-111629 (QP Corp.), JP-A 63-258528 (QP Corp.) or EP 426211 (Unilever).

It is particularly advantageous to use the variant together with an anti-staling endo-amylase and optionally also to add a phospholipid, to reduce-staling of the bread and particularly to improve softness of the bread in the first 24 hours after baking. The endo-amylase may be a maltogenic α-amylase (e.g. from *Bacillus sp.*, such as Novamyl® from Novo Nordisk) or a fungal or bacterial α-amylase, e.g. from *Aspergillus* or *Bacillus*, particularly *A. oryzae*, *B. licheniformis* or *B. amyloliquefaciens*.

Use of variant in detergent

The variant of the invention has phospholipase activity and may be used as an ingredient of a detergent composition. The variant is preferably used at a concentration (expressed as pure enzyme protein) of 0.001-10 (e.g. 0.01-1) mg per gram of detergent or 0.001-100 (e.g. 0.01-10) mg per liter of wash liquor.

Preferred variants for use in a detergent have phospholipase activity at alkaline pH, and preferably also have lipase activity. The following lists some preferred variants for use in detergents:

E1SPPCGRRP +E99N +E239C +Q249R +G266D
E1SPPCGRRP +E239C +Q249R +G266D
E1SPPCGRRP +L93K +E99K +E239C +Q249R +G266D
E1SPPCGRRP +E99K +E239C +Q249R +G266D
G266A
G266W
G266E
G266V
G263Q +L264A +I265T +G266D +T267A
G263F +L264A +G266S +T267E
E1SPPCGRRP +E239C +Q249R +G263Q +L264A +I265T +G266D +T267A
G266S
G266K
G266L
G266R
G263A +G266A
G263A +G266Y
E1SPPCGRRP +E239C +Q249R +G266A
E1SPPCGRRP +E239C +Q249R +G266S
E1SPPCGRRP +E239C +Q249R +G263F +L264A +G266S +T267E

D62A + G266A
D62A + G266S
D96S + G266A
D96S+ G266S
D96S+ G266R
D96S+ G266W
D96S+ G266V

Detergent composition

The detergent composition of the invention may for example be formulated as a hand or machine laundry detergent composition including a laundry additive composition suitable for pre-treatment of stained fabrics and a rinse added fabric softener composition, or be formulated as a detergent composition for use in general household hard surface cleaning operations. In a laundry detergent, the variant may be effective for the removal of fatty stains, for whiteness maintenance and for dingy cleanup. A laundry detergent composition may be formulated as described in WO 97/04079, WO 97/07202, WO 97/41212, PCT/DK WO 98/08939 and WO 97/43375.

The detergent composition of the invention may particularly be formulated for hand or machine dishwashing operations. e.g. as described in GB 2,247,025 (Unilever) or WO 99/01531 (Procter & Gamble). In a dishwashing composition, the variant may be effective for removal of greasy/oily stains, for prevention of the staining /discoloration of the dishware and plastic components of the dishwasher by highly colored components and the avoidance of lime soap deposits on the dishware.

The detergent composition of the invention may be in any convenient form, e.g., a bar, a tablet, a powder, a granule, a paste or a liquid. A liquid detergent may be aqueous, typically containing up to 70 % water and 0-30 % organic solvent, or non-aqueous.

The detergent composition comprises one or more surfactants, which may be non-ionic including semi-polar and/or anionic and/or cationic and/or zwitterionic. The surfactants are typically present at a level of from 0.1% to 60% by weight, e.g. 0.5-40 %, preferably 1-30 %, typically 1.5-20 %.

When included therein the detergent will usually contain from about 1% to about 40% of an anionic surfactant such as linear alkylbenzenesulfonate, alpha-olefinsulfonate, alkyl sulfate (fatty alcohol sulfate), alcohol ethoxysulfate, secondary alkanesulfonate, alpha-sulfo fatty acid methyl ester, alkyl- or alkenylsuccinic acid or soap.

When included therein the detergent will usually contain from about 0.2% to about 40% of a non-ionic surfactant such as alcohol ethoxylate, nonyl-phenol ethoxy-

late, alkylpolyglycoside, alkyldimethylamine-oxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, polyhydroxy alkyl fatty acid amide, or N-acyl N-alkyl derivatives of glucosamine ("glucamides").

The invention also provides a detergent additive comprising the variant of the invention. The detergent additive as well as the detergent composition may comprise one or more other enzymes such as a protease, a lipase, a cutinase, an amylase, a carbohydrase, a cellulase, a pectinase, a mannanase, an arabinase, a galactanase, a xylanase, an oxidase, e.g., a laccase, and/or a peroxidase.

In general the properties of the chosen enzyme(s) should be compatible with the selected detergent, (i.e. pH-optimum, compatibility with other enzymatic and non-enzymatic ingredients, etc.), and the enzyme(s) should be present in effective amounts.

Proteases: Suitable proteases include those of animal, vegetable or microbial origin. Microbial origin is preferred. Chemically modified or protein engineered mutants are included. The protease may be a serine protease or a metallo protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from Bacillus, e.g., subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270 and WO 94/25583.

Examples of useful proteases are the variants described in WO 92/19729, WO 98/20115, WO 98/20116, and WO 98/34946, especially the variants with substitutions in one or more of the following positions: 27, 36, 57, 76, 87, 97, 101, 104, 120, 123, 167, 170, 194, 206, 218, 222, 224, 235 and 274.

Preferred commercially available protease enzymes include Alcalase ®, Savinase ®, Primase ®, Duralase ®, Esperase ®, and Kannase ® (Novo Nordisk A/S), Maxatase®, Maxacal®, Maxapem®, Properase®, Purafect®, Purafect OxP®, FN2™, and FN3™ (Genencor International Inc.).

Lipases: Suitable lipases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful lipases include lipases from *Humicola* (synonym *Thermomyces*), e.g. from *H. lanuginosa* (*T. lanuginosus*) as described in EP 258 068 and EP 305 216 or from *H. insolens* as described in WO 96/13580, a *Pseudomonas* lipase, e.g. from *P. alcaligenes* or *P. pseudoalcaligenes* (EP 218 272), *P. cepacia* (EP 331 376), *P. stutzeri* (GB 1,372,034), *P. fluorescens*, *Pseudomonas sp.* strain SD 705 (WO 95/06720 and WO 96/27002), *P. wisconsinensis* (WO 96/12012), a *Bacillus* lipase, e.g. from *B. subtilis* (Dartois et al. (1993), Biochemica et Biophysica Acta, 1131, 253-360), *B. stearothermophilus* (JP 64/744992) or *B. pumilus* (WO 91/16422).

Other examples are lipase variants such as thos described in WO 92/05249, WO 94/01541, EP 407 225, EP 260 105, WO 95/35381, WO 96/00292, WO 95/30744, WO 94/25578, WO 95/14783, WO 95/22615, WO 97/04079 and WO 97/07202.

Preferred commercially available lipase enzymes include Lipolase™ and Lipolase Ultra™ (Novo Nordisk A/S).

Cellulases: Suitable cellulases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Suitable cellulases include cellulases from the genera *Bacillus, Pseudomonas, Humicola, Fusarium, Thielavia, Acremonium*, e.g. the fungal cellulases produced from Humicola insolens, *Myceliophthora thermophila* and *Fusarium oxysporum* disclosed in US 4,435,307, US 5,648,263, US 5,691,178, US 5,776,757 and WO 89/09259.

Especially suitable cellulases are the alkaline or neutral cellulases having colour care benefits. Examples of such cellulases are cellulases described in EP 0 495 257, EP 0 531 372, WO 96/11262, WO 96/29397, WO 98/08940. Other examples are cellulase variants such as those described in WO 94/07998, EP 0 531 315, US 5,457,046, US 5,686,593, US 5,763,254, WO 95/24471, WO 98/12307 and PCT/DK98/00299.

Commercially available cellulases include Celluzyme®, and Carezyme® (Novo Nordisk A/S), Clazinase®, and Puradax HA® (Genencor International Inc.), and KAC-500(B)® (Kao Corporation).

Peroxidases/Oxidases: Suitable per-oxidases/oxidases include those of plant, bac-terial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful peroxidases include peroxidases from *Coprinus*, e.g. from *C. cinereus*, and variants thereof as those described in WO 93/24618, WO 95/10602, and WO 98/15257.

Commercially available peroxidases include Guardzyme® (Novo Nordisk A/S).

The detergent enzyme(s) may be included in a detergent composition by adding separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes. A detergent additive of the invention, i.e. a separate additive or a combined additive, can be formulated e.g. as a granulate, a liquid, a slurry, etc. Preferred detergent additive formulations are granulates, in particular non-dusting granulates, liquids, in particular stabilized liquids, or slurries.

Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molar weights of 1000 to 20000; ethoxylated nonyl-phenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alco-

hols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in GB 1483591. Liquid enzyme preparations may, for in-stance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent may contain 0-65 % of a detergent builder or complexing agent such as zeolite, diphosphate, tripho-sphate, phosphonate, carbonate, citrate, nitrilotriacetic acid, ethylenediaminetetraacetic acid, diethylenetri-aminepen-taacetic acid, alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst).

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose, poly(vinyl-pyrrolidone), poly (ethylene glycol), poly(vinyl alcohol), poly(vinylpyridine-N-oxide), poly(vinylimidazole), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid co-polymers.

The detergent may contain a bleaching system which may comprise a H2O2 source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine or nonanoyloxybenzenesul-fonate. Alternatively, the bleaching system may comprise peroxyacids of e.g. the amide, imide, or sulfone type.

The enzyme(s) of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g., a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative, e.g., an aromatic borate ester, or a phenyl boronic acid derivative such as 4-formylphenyl boronic acid, and the composition may be formulated as described in e.g. WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as e.g. fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil redeposition agents, dyes, bactericides, optical brighteners, hydrotropes, tarnish inhibitors, or perfumes.

It is at present contemplated that in the detergent compositions any enzyme, in particular the variant of the invention, may be added in an amount corresponding to 0.01-100 mg of enzyme protein per liter of wash liquor, preferably 0.05-5 mg of enzyme protein per liter of wash liquor, in particular 0.1-1 mg of enzyme protein per liter of wash liquor.

The variant of the invention may additionally be incorporated in the detergent formulations disclosed in WO 97/07202 which is hereby incorporated as reference.

Methods for preparing enzyme variants

The enzyme variant of the invention can be prepared by methods known in the art, e.g. as described in WO 97/04079 (Novo Nordisk). The following describes methods for the cloning of enzyme-encoding DNA sequences, followed by methods for generating mutations at specific sites within the enzyme-encoding sequence.

Cloning a DNA sequence encoding a enzyme

The DNA sequence encoding a parent enzyme may be isolated from any cell or microorganism producing the enzyme in question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the enzyme to be studied. Then, if the amino acid sequence of the enzyme is known, labeled oligonucleotide probes may be synthesized and used to identify enzyme-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labeled oligonucleotide probe containing sequences homologous to another known enzyme gene could be used as a probe to identify enzyme-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying enzyme-encoding clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming enzyme-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for enzyme (*i.e.* maltose), thereby allowing clones expressing the enzyme to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, *e.g.* the phosphoroamidite method described S.L. Beaucage and M.H. Caruthers, (1981), Tetrahedron Letters 22, p. 1859-1869, or the method described by Matthes et al., (1984), EMBO J. 3, p. 801-805. In the phosphoroamidite method, oligonucleotides are synthesized, *e.g.* in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al., (1988), Science 239, 1988, pp. 487-491.

Site-directed mutagenesis

Once a enzyme-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites. In a specific method, a single-stranded gap of DNA, the enzyme-encoding sequence, is created in a vector carrying the enzyme gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al., (1984), Biotechnology 2, p. 646-639. US 4,760,025 discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method for introducing mutations into enzyme-encoding DNA sequences is described in Nelson and Long, (1989), Analytical Biochemistry 180, p. 147-151. It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Further, Sierks. et al., (1989) "Site-directed mutagenesis at the active site Trp120 of *Aspergillus awamori* glucoamylase. Protein Eng., 2, 621-625; Sierks et al., (1990), "Catalytic mechanism of fungal glucoamylase as defined by mutagenesis of Asp176, Glu179 and Glu180 in the enzyme from *Aspergillus awamori*". Protein Eng. vol. 3, 193-198; also describes site-directed mutagenesis in an *Aspergillus* glucoamylase.

Expression of enzyme variants

According to the invention, a DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

Expression vector

The recombinant expression vector carrying the DNA sequence encoding a enzyme variant of the invention may be any vector which may conveniently be subjected

to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. The vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated. Examples of suitable expression vectors include pMT838.

Promoter

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the DNA sequence encoding a enzyme variant of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dag*A promoters, the promoters of the *Bacillus licheniformis* α-amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* α-amylase (*amyQ*), the promoters of the *Bacillus subtilis* xylA and xylB genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, the TPI (triose phosphate isomerase) promoter from *S. cerevisiae* (Alber et al. (1982), J. Mol. Appl. Genet 1, p. 419-434, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

Expression vector

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the α-amylase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the v ctor may comprise *As-*

pergillus selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the s lection may be accomplished by co-transformation, *e.g.* as described in WO 91/17243.

The procedures used to ligate the DNA construct of the invention encoding a enzyme variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989).

Host Cells

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of a enzyme variant of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, *e.g.* by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are Gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gramnegative bacteria such as *E.coli*. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The yeast organism may favorably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, *e.g. Saccharomyces cerevisiae*.

The host cell may also be a filamentous fungus *e.g.* a strain belonging to a species of *Aspergillus*, most preferably *Aspergillus oryzae* or *Aspergillus niger, or* a strain of *Fusarium*, such as a strain of *Fusarium oxysporium*, *Fusarium graminearum* (in the perfect state named *Gribberella zeae,* previously *Sphaeria zeae, synonym with Gibberella roseum and Gibberella roseum* f. sp. *cerealis)*, or *Fusarium sulphureum (*in the prefect

stat named *Gibberella puricaris*, synonym with *Fusarium trichothecioides, Fusarium bactridioides, Fusarium sambucium, Fusarium roseum,* and *Fusarium roseum var. graminearum), Fusarium cerealis* (synonym with *Fusarium crokkwellnse), or Fusarium venenatum.*

In a preferred embodiment of the invention the host cell is a protease deficient of protease minus strain.

This may for instance be the protease deficient strain *Aspergillus oryzae* JaL 125 having the alkaline protease gene named "alp" deleted. This strain is described in WO 97/35956 (Novo Nordisk).

Filamentous fungi cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. The use of *Aspergillus* as a host micro-organism is described in EP 238 023 (Novo Nordisk A/S), the contents of which are hereby incorporated by reference.

Method of producing the enzyme variant of the invention

The enzyme variant of the invention may be produced by a method comprising cultivating a host cell under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the enzyme variant of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (*e.g.* as described in catalogues of the American Type Culture Collection).

The enzyme variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Expression of variant in plants

The present invention also relates to a transgenic plant, plant part or plant cell which has been transformed with a DNA sequence encoding the variant of the invention so as to express and produce this enzyme in recoverable quantities. The enzyme may be recovered from the plant or plant part. Alternatively, the plant or plant part containing th recombinant nzyme may be used as such.

The transgenic plant can b dicotyledonous or monocotyledonous, for short a dicot or a monocot. Examples of monocot plants are grasses, such as meadow grass (blue grass, Poa), forage grass such as festuca, lolium, temperate grass, such as Agrostis, and cereals, e.g. wheat, oats, rye, barley, rice, sorghum and maize (corn).

Examples of dicot plants are tobacco, legumes, such as lupins, potato, sugar beet, pea, bean and soybean, and cruciferous (family Brassicaceae), such as cauliflower, oil seed rape and the closely related model organism Arabidopsis thaliana.

Examples of plant parts are stem, callus, leaves, root, fruits, seeds, and tubers. In the present context, also specific plant tissues, such as chloroplast, apoplast, mitochondria, vacuole, peroxisomes and cytoplasm are considered to be a plant part. Furthermore, any plant cell, whatever the tissue origin, is considered to be a plant part.

Also included within the scope of the invention are the progeny of such plants, plant parts and plant cells.

The transgenic plant or plant cell expressing the variant of the invention may be constructed in accordance with methods known in the art. In short the plant or plant cell is constructed by incorporating one or more expression constructs encoding the variant of the invention into the plant host genome and propagating the resulting modified plant or plant cell into a transgenic plant or plant cell.

Conveniently, the expression construct is a DNA construct which comprises a gene encoding the variant of the invention in operable association with appropriate regulatory sequences required for expression of the gene in the plant or plant part of choice. Furthermore, the expression construct may comprise a selectable marker useful for identifying host cells into which the expression construct has been integrated and DNA sequences necessary for introduction of the construct into the plant in question (the latter depends on the DNA introduction method to be used).

The choice of regulatory sequences, such as promoter and terminator sequences and optionally signal or transit sequences is determined, eg on the basis of when, where and how the enzyme is desired to be expressed. For instance, the expression of the gene encoding the variant of the invention may be constitutive or inducible, or may be developmental, stage or tissue specific, and the gene product may be targeted to a specific tissue or plant part such as seeds or leaves. Regulatory sequences are eg described by Tague et al, Plant, Phys., 86, 506, 1988.

For constitutive expression the 35S-CaMV promoter may be used (Franck et al., 1980. Cell 21: 285-294). Organ-specific promoters may eg be a promoter from storage sink tissues such as seeds, potato tubers, and fruits (Edwards & Coruzzi, 1990. Annu. Rev. Genet. 24: 275-303), or from metabolic sink tissues such as meristems (Ito et al., 1994. Plant Mol. Biol. 24: 863-878), a seed specific promoter such as the glutelin, prolamin, globulin or albumin promoter from rice (Wu et al., Plant and Cell Physiology

Vol. 39, No. 8 pp. 885-889 (1998)), a *Vicia faba* promoter from the legumin B4 and the unknown seed protein gene from *Vicia faba* described by Conrad U. et al, Journal of Plant Physiology Vol. 152, No. 6 pp. 708-711 (1998), a promoter from a seed oil body protein (Chen et al., Plant and cell physiology vol. 39, No. 9 pp. 935-941 (1998), the storage protein napA promoter from Brassica napus, or any other seed specific promoter known in the art, eg as described in WO 91/14772. Furthermore, the promoter may be a leaf specific promoter such as the rbcs promoter from rice or tomato (Kyozuka et al., Plant Physiology Vol. 102, No. 3 pp. 991-1000 (1993), the chlorella virus adenine methyltransferase gene promoter (Mitra, A. and Higgins, DW, Plant Molecular Biology Vol. 26, No. 1 pp. 85-93 (1994), or the aldP gene promoter from rice (Kagaya et al., Molecular and General Genetics Vol. 248, No. 6 pp. 668-674 (1995), or a wound inducible promoter such as the potato pin2 promoter (Xu et al, Plant Molecular Biology Vol. 22, No. 4 pp. 573-588 (1993).

A promoter enhancer element may be used to achieve higher expression of the enzyme in the plant. For instance, the promoter enhancer element may be an intron which is placed between the promoter and the nucleotide sequence encoding the enzyme. For instance, Xu et al. *op cit* disclose the use of the first intron of the rice actin 1 gene to enhance expression.

The selectable marker gene and any other parts of the expression construct may be chosen from those available in the art.

The DNA construct is incorporated into the plant genome according to conventional techniques known in the art, including *Agrobacterium*-mediated transformation, virus-mediated transformation, micro injection, particle bombardment, biolistic transformation, and electroporation (Gasser et al, Science, 244, 1293; Potrykus, Bio/Techn. 8, 535, 1990; Shimamoto et al, Nature, 338, 274, 1989).

Presently, *Agrobacterium tumefaciens* mediated gene transfer is the method of choice for generating transgenic dicots (for review Hooykas & Schilperoort, 1992. Plant Mol. Biol. 19: 15-38), however it can also be used for transforming monocots, although other transformation methods are generally preferred for these plants. Presently, the method of choice for generating transgenic monocots is particle bombardment (microscopic gold or tungsten particles coated with the transforming DNA) of embryonic calli or developing embryos (Christou, 1992. Plant J. 2: 275-281; Shimamoto, 1994. Curr. Opin. Biotechnol. 5: 158-162; Vasil et al., 1992. Bio/Technology 10: 667-674). An alternative method for transformation of monocots is based on protoplast transformation as described by Omirulleh S, et al., Plant Molecular biology Vol. 21, No. 3 pp. 415-428 (1993).

Following transformation, the transformants having incorporated the expression construct are selected and regenerated into whole plants according to methods well-known in the art.

MATERIALS AND METHODS

Lipase activity (LU)

A substrate for lipase is prepared by emulsifying tributyrin (glycerin tributyrate) using gum Arabic as emulsifier. The hydrolysis of tributyrin at 30 °C at pH 7 is followed in a pH-stat titration experiment. One unit of lipase activity (1 LU) equals the amount of enzyme capable of releasing 1 µmol butyric acid/min at the standard conditions.

Phospholipase activity (PHLU)

Phospholipase activity (PHLU) is measured as the release of free fatty acids from lecithin. 50 µl 4% L-alpha-phosphatidylcholine (plant lecithin from Avanti), 4 % Triton X-100, 5 mM $CaCl_2$ in 50 mM HEPES, pH 7 is added 50 µl enzyme solution diluted to an appropriate concentration in 50 mM HEPES, pH 7. The samples are incubated for 10 min at 30 °C and the reaction stopped at 95 °C for 5 min prior to centrifugation (5 min at 7000 rpm). Free fatty acids are determined using the NEFA C kit from Wako Chemicals GmbH; 25 µl reaction mixture is added 250 µl Reagent A and incubated 10 min at 37 °C. Then 500 µl Reagent B is added and the sample is incubated again, 10 min at 37 °C. The absorption at 550 nm is measured using an HP 8452A diode array spectrophotometer. Samples are run in at least in duplicates. Substrate and enzyme blinds (preheated enzyme samples (10 min at 95 °C) + substrate) are included. Oleic acid is used as a fatty acid standard. 1 PHLU equals the amount of enzyme capable of releasing 1 µmol of free fatty acid/min at these conditions.

Phospholipase monolayer assay

On a thoroughly purified surface of a buffer solution (either 10 mM Glycin, pH 9.0 or 10 mM NaOAc, pH 5.0; 1 mM CaCl2, 25°C) a monolayer of Di-Decanoyl-Phosphatidyl Choline (DDPC) is spread from a chloroform solution. After relaxation of the monolayer (evaporation of chlorofom) the surface pressure is adjusted to 15 mN/m, corresponding to a mean molecular area of DDPC of approx. 63 $Å^2$/molec. A solution containing approximately 60 µg (micro gram) enzyme is injected through the monolayer into the subphase of the re-action compartment (cylinder with surface area 2230 mm2 and reaction volume 56570 mm3) in the "zero-order trough". Enzymatic activity is manifested through the speed of a mobil barrier compressing the monolayer in order

to maintain constant surface pressure as insoluble substrate molecules are hydrolysed into more water soluble reaction products. Having verified that the aqueous solubility of the reaction products (capric acid and MDPC) are considerably higher than for DDPC the number of DDPC-molecules hydrolyzed per minute by the enzyme is estimated from the mean molecular area (MMA) of DDPC. The results are calculated on basis of average barrier speed over the first 5 minutes of hydrolysis.

Yeast Strain

Saccharomyces cerevisiae YNG318: MATa leu2-D2 ura3-52 his4-539 pep4-D1[cir+] , described in WO 97/04079 and WO 97/07205.

Transformation of yeast strain

The DNA fragments and the opened vectors are mixed and transformed into the yeast *Saccharomyces cerevisiae* YNG318 by standard methods.

Vector for yeast transformation

pJSO026 (*S. cerevisiae* expression plasmid) is described in WO 97/07205 and in J.S.Okkels, (1996) "A URA3-promoter deletion in a pYES vector increases the expression level of a fungal lipase in Saccharomyces cerevisiae. Recombinant DNA Biotechnology III: The Integration of Biological and Engineering Sciences, vol. 782 of the Annals of the New York Academy of Sciences). It is derived from pYES 2.0 by replacing the inducible GAL1-promoter of pYES 2.0 with the constitutively expressed TPI (triose phosphate isomerase)-promoter from *Saccharomyces cerevisiae* (Albert and Karwasaki, (1982), J. Mol. Appl Genet., 1, 419-434), and deleting a part of the URA3 promoter.

Screening method

The yeast libraries are spread on cellulose filters on SC-ura agar plates and incubated for 3-4 days at 30°C.

The filters are then transferred to the lecithin plates and incubated at 37°C for 2-6 hours. Yeast cells harbouring active phospholipases will develope white clearing zones around the colonies. The positive variants can then be further purified and tested.

Media

SC-ura medium

Yeast Nitrogen (without amino aicds)	7.5 g
Succinic acid	11.3 g
NaOH	6.8 g
Casaminoacid (without vitamins)	5.6 g
Tryptophan	0.1 g
Agar, Merck	20 g
Distilled water	ad 1000 ml

Autoclaved for 20 minutes at 121°C.

From a sterile stock solution of 5% Threonine 4 ml is added to a volume of 900 ml together with 100 ml of a sterile 20% glucose.

Preparation of Lecithin plates:

10 g agarose is melted in 550 ml H2O by boiling in a microwave oven. After cooling to 60-70°C the following ingredients are added:

250 ml of a 0.4 M Citrate buffer (pH 4.5 or pH 7.1)

200 ml 3% lecithin (from Avanti) in 2% Triton-X 100

2 ml 2% crystal violet

30 ml of the mixture is poured into 14 cm Ø petri dishes.

EXAMPLES

Example 1: Construction of variants with Lipolase "backbone" and C-terminal from *Fusarium oxysporum* phospholipase by PCR reaction

For the Lipolase backbone the following variants were used as templates: E1A +G91A +D96W +E99K +Q249R and SPIRR +G91A +D96W +E99K +Q249R. Wild-type Lipolase was used for generating a fragment in the C-terminal without Q249R. The template for the C-terminal phospholipase was the F.o. phospholipase, cloned in the same vector as the Lipolase variants.

PCR reaction 1: 4244 as 5' primer and H7 as 3'primer and one of the two templates mentioned above.

PCR reaction 2: FOL14 as 5' primer and FOL15 as 3' primer and wild-type Lipolase as template (no mutation in pos 249)

PCR reaction 3: FOL16 as 5' primer and AP as 3' primer and F.o. phospholipase as template

A PCR reaction 4 was made to create the connection between the Lipolase variant and the C-terminal from the phospholipase by using FOL14 as 5' primer and AP as 3' primer and PCR reaction 2 and 3 as template.

The final PCR was made with 4244 as 5' primer and KBoj14 as 3' primer and PCR re-action 1 and 4 as template. (By using wild-type Lipolase as template in reaction 2 a possibility to omit the mutation in position 249 was created).

The final PCR fragment was used in an in vivo recombination in yeast together with pJSO026 cut with the restriction enzymes. SmaI(or BamHI) and XbaI (to remove the coding region and at the same time create an overlap of about 75 bp in each end to make a recombination event possible). This final treatment was also used in the following examples.

Primer FOL14 and primer 15/16 are mixed oligoes to give the possibility to bind both with Lipolase and phospholipase templates and at the same time give possibilities for introducing the amino acids from both templates in the different positions. For some of the positions new amino acids could be introduced as well.

Primer FOL14

Position 205 in the *H. lanuginosa* lipase: 75% R, 25% S

Primer FOL15/16

Position 256 in the *H. lanuginosa* lipase: 50% P, 50% A

Position 260 in the *H. lanuginosa* lipase: 25% R, 12.5% Q, 12.5% H, 12.5% C, 12.5% Y, 12.5% W, 12.5% stop.

The sequences of the resulting variants were determined, and were found to correspond to Lipolase with the following alterations. Alterations in parentheses are uncertain.

E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

E1A, G91A, D96W, E99K, E239C, Q249R, P256A, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G273F, (274S)

E1A, G91A, D96W, E99K, N248T, Q249R, W260Q, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

SPIRR, G91A, D96W, E99K, W260C, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272, G273F, (274S)

SPIRR, G91A, D96W, E99K, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

E1A, G91A, D96W, E99K, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

Exampl 2: Production of truncated sequences

Variants were made with stop after amino acid 269, 270, 271, 272,(273 and 274)

The following PCR reactions were made with the following template: E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S).

Reaction 1: 5' primer 4244 and 3' primer KBoj36 (stop after 269)

Reaction 2: 5' primer 4244 and 3' primer KBoj37 (stop after 270)

Reaction 3: 5' primer 4244 and 3' primer KBoj38 (stop after 271)

Reaction 4: 5' primer 4244 and 3' primer KBoj39 (stop after 272)

The sequences of the resulting variants were determined, and were found to correspond to Lipolase with the following alterations:

E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N

E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A

E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G

E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G

Example 3: Removal of mutations in the lid region

G91A or E99K can be removed without loosing the phospholipase activity. The sequences of the resulting variants were determined, and were found to correspond to Lipolase with the following alterations:

E1A, G91A, D96W, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

SPIRR, D96W, E99K, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

SPIRR, G91A, D96W, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

E1A, G91A, D96W, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

Example 4: Doping in the C-terminal region of Lipolase to introduce phospholipas activity

Three different libraries were constructed with possibilities for mutations in position 256 and position 263-269. At the same time possibilities for extension of the C-terminal with either 1, 2 , 3 or 4 amino acids were included.

Doping, the wt sequences are underlined:

256: P 94, A 3, T 3

263: G 87, E 4.8, A 3.8, R 3.6, Q 0.2, P 0.2

264: L 87, P 4.8, Q 3.8, V 3.6, A 0.2, E 0.2

265: I 85, T 5.6, L 2.2, S 1.6, N 1.5, F 1.4, R 0.4, K 0.4 A,P 0.1, G,D,C,H,Y 0.03, Q,E 0.01, stop 0.016

266: G 86, D 5.9, R 2, S 1.7, C 1.6, A 0.9, V 0.9, E 0.7, W 0.2, H,Y 0.1, I,L,T,F,P 0.02, Q,K 0.01, stop 0.014

267: T 86, A 6.6, S 1.9, R 0.9, N 0.9, I 0.9, K 0.9, M 0.9, P 0.9, P 0.9, G,V 0.14, D,E 0.07, L 0.03, C,Q,H,F,W,Y 0.01, stop 0.01

268: C 91, S 1.9, R 1.0, G 1.0, F 0.9, Y 0.9, L 0.04, A,N,D,H,I,P,T,V 0.01, stop 2.8

269: L 92, stop 8 (KBoj 32 and KBoj33)/ N 86, K 2.7, D 1.8, H 1.8, I 1.8, S 1.8, T 1.9, Y 1.8, R 0.1, Q,M,E 0.06, A,C,G,L,F,P,V 0.04, stop 0.06(KBoj34)

270: stop 100 (KBoj33)/A 44, P 44, S 1.9, T 1.8, R 1.5, L 1.5, G 1.4, V 1.4, D 0.7, Q 0.7, E 0.7, H 0.7, N,C,I,K,M,F,W,Y 0.03, stop 0.03 (KBoj 32 and KBoj 34)

271: G 72, R 4.5, V 3.2, E 3.0, C 2.9, A 1.6, S 1.2, D 1.0, L 0.5, I,K,Y 0.15, Q,T 0.08, N,P 0.05, stop 9.2

272: G 72, R 4.5, V 3.2, E 3.0, C 2.9, A 1.6, S 1.2, D 1.0, L 0.5, I,K,Y 0.15, Q,T 0.08, N,P 0.05, stop 9.2

273: F 74, L 11, S 2.8, I 2.7, V 2.7, Y 2.5, C 2.5, A,R,T 0.1, N,D,H 0.08, Q,E,K 0.01, stop 0.5

274 STOP

Library A: PCR reaction with 4244 as 5' primer and KBoj 33 as 3' primer and E1A +G91A +D96W +E99K +Q249R or E1A +G225R as template. Variants from this library will be without extension.

Library B: PCR reaction with 4244 as 5' primer and KBoj 32 as 3' primer and E1A +G91A +D96W +E99K +Q249R or E1A +G225R as template. Variants from this library will most probably contain a C-terminal extension but can contain stop codons before the extension.

Library C: PCR reaction with 4244 as 5' primer and KBoj 34 as 3' primer and E1A +G91A +D96W +E99K +Q249R or E1A +G225R as template. Variants from this

library will most probably contain mutations in position 269 and a C-terminal extension but can contain stop codons before the extension.

The following variants were obtained:

Library A:

E1A +G91A +D96W +E99K +Q249R +G266D

Library B:

E1A +G91A +D96W +E99K +(R232L) +Q249R +G266S +270A
E1A +G91A +D96W +E99K +Q249R +G266S +270D +271G
E1A+ G91A+ D96W+ E99K+ Q249R+ L264G+ I265G+ G266F+ T267stop
E1A +G91A +D96W +E99K +Q249R +G266A +270P +271G
E1A +G91A +D96W +E99K +Q249R +L264P +I265F +L269stop

Library C:

E1A +G91A +D96W +E99K +Q249R +G263E +G266D +L269N +270P +271V +272G +273F
E1A +G91A +D96W +E99K +Q249R +G263A +G266S +L269N +270A +271G +272R +273F
E1A +G91A +D96W +E99K +Q249R +L264P -G266 +L269I +270P +271R +272G +273F
E1A +G91A +D96W +E99K +Q249R +G266D +L269S +270A +271G +272G +273F
E1A +D27G +G91A +D96W +E99K +Q249R +G266S +L269N +270A +271G +272G +273F
E1A +G91A +D96W +E99K +Q249R +G266D +L269N +270A
E1A +G91A +D96W +E99K +Q249R +L264P +L267Q +L269N
E1A +G91A +D96W +E99K +Q249R +G263R +I265L +L269N +270P

Example 5: Lipase and phospholipase activities of variants

A number of variants of the invention (described above) were purified and analyzed as follows. A prior-art enzyme from *F. oxysporum* known to have both lipase and phospholipase activity was included for comparison. The results are shown in the table below.

The lipase (LU) and phospholipase (PHLU) activity was measured and expressed as activity per mg of pure enzyme protein (measured by absorption at nm). The ratio of phospholipase to lipase is also shown in the table. Further, the pH optimum was determined by using the LU and PHLU methods at various pH values.

	Variant (ee f otnotes)	Activity		Rati	pH ptimum	
		LU/A_{280}	PHLU/A_{280}	PHLU / LU	LU	PHLU
Invention	*1)	24	173	7.2	9-10	6.0
	*2)	194	824	4.2	9-10	4 .0
	*3)	138	424	3.07		4.0
	*4)	27	229	8.48		4.0
	*5)	1568				4
	*6)				6	
	*7)				10	
	*8)				6	5
Prior art	*F. oxysporum* wild type	2500	2500	1.0	9-10	9-10

*1) E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

*2) SPIRR, G91A, D96W, E99K, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

*3) E1A, G91A, D96W, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

*4) E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N

*5) E1A, G91A, D96W, E99K, Q249R, G266S, 270D, 271G

*6) E1A, G91A, D96W, E99K, Q249R, G266D

*7) E1A, G91A, D96W, E99K, Q249R, G266A, 270P, 271G

*8) G266D

Example 6: Phospholipase activity by monolayer assay

A number of variants were analyzed for phospholipase activity by the mono layer assay described above at pH 5 and 9. Some prior-art lipases and variants were included as reference. The results (in nmol/min) were as follows (FoL refers to the *Fusarium oxysporum* lipase/phospholipase):

	Variant (see footnotes in Example 5)	pH 5	pH 9
Invention	*1)	4.5	3.4
	*2)	4.8	6.3
	*3)	6.2	7.5
	*4)	5.8	5.3
	*5)	1.3	2.6
	*6)	3.3	0.1
	*7)	0.5	0.2
	*8)	0.20	0.3
Prior art	Lipolase	0.0	0.0
	Lipolase variant: SPIRR +N94K +F95L +D96H +N101S +F181L +D234Y +I252L +P256T +G263A +L264Q	0.0	
	FoL	3.6	5.2

Example 7: Thermostability of variants

The thermostability of variants was tested by DSC (Differential Scanning Calorimetry) by heating at 90 deg/hr at pH 5 (50 mM acetate buffer), pH 9 (50 mM borate buffer) or pH 10 (50 mM glycine buffer). The temperature at the top of the denaturation peak (T_d) was found as follows:

	Variant (see footnotes in Example 5)	pH 5	pH 9	pH 10
Invention	*1)	54°C	62°C	
	*2)	57°C	57°C	56°C
	*3)	58°C	68°C	68°C
	*5)		64°C	61°C
	*8)	69°C	65°C	67°C
Prior art	*Fusarium oxysporum* lipase		49°C	

Example 8: D gumming of vegetable oil

Rape seed oil was treated with two variants of the invention, essentially as described in Example 6 of WO 98/18912 (Novo Nordisk).

The variant shown in footnote *1) of Example 5 was tested at various pH and temperatures. The enzyme dosage was 0.6 mg of enzyme protein per kg of oil. Results:

Temp.	pH	Initial performance	Final P content after 6 hours
45 °C	4.5	0 %	42
45 °C	5.0	40 %	11
45 °C	5.7	100 %	4
45 °C	6.5	60 %	3
35 °C	5.8	90 %	4
50 °C	5.5	0 %	39
55 °C	5.5	0 %	38

The results show optimum performance at pH 5.7, 35-45°C. A separate experiment at 45°C, pH 6 showed that a final P content of 4 ppm could be reached at an enzyme dosage as low as 0.15 mg/kg.

A similar experiment with the variant shown in footnote *2) of Example 5 showed optimum performance at 40°C, pH 5.0-5.5. The enzyme dosage was 0.3 mg/kg.

A degumming experiment was made with the variant shown in footnote 8 of Example 5, using rape seed oil at 45°C, pH 5, 1.8 mg enzyme/kg oil. For comparison, a similar experiment was made with the parent lipase (Lipolase) at 18 mg/kg. The results (P content) were as follows:

	Invention	Reference
	Variant	Lipolase
0 hours	166 ppm	231 ppm
3.4 hours	7 ppm	203 ppm

The results show that good degumming (<10 ppm residual P content) was obtained in 3.4 hours with the variant. The parent lipase had very little degumming effect, even at 10 times higher enzyme dosage.

Example 9: Baking tests

The variant shown in footnote *1) of Example 5 was evaluated in baking tests as follows.

Doughs were prepared from Meneba flour according to the European straight dough method (ABF-SP-1201.01). The variant, ascorbic acid, phospholipase (lecithin) and endo-amylase were added to the dough as described below. The endo-amylase was maltogenic amylase from *B. stearothermophilus* (tradename Novamyl®). The amylase dosage is given in MANU units. One MANU (Maltogenic Amylase Novo Unit) may be defined as the amount of enzyme required to release one μmol of maltose per minute at a concentration of 10 mg of maltotriose substrate per ml of 0.1 M citrate buffer, pH 5.0 at 37 °C for 30 minutes.

Dough	Ascorbic acid	Variant mg enzyme/kg	Phospholipase mg enzyme/kg	Lecithin g/ kg	Novamyl MANU/kg
1	40 ppm	0	0	0	0
2	40 ppm	0	0.4	10	0
3	40 ppm	0.25	0	10	0
4	40 ppm	0.5	0	10	0
5	40 ppm	1.5	0	10	0
6	40 ppm	0	0	10	750
7	40 ppm	1.5	0	10	750
8	40 ppm	0.25	0	10	750

After baking, the loaves were cooled, and the loaf volume, crumb firmness and softness were evaluated after approximately 2 hours. The evaluation was repeated after 1, 3 and 7 days storage at 22°C wrapped in double plastic bags.

Firmness of crumb was measured using a texture analyzer TA-XT2 from Stable Micro Systems (probe diameter 40 mm).

Softness in gram was measured as the force needed to press a probe 6,25 mm into a crumb of a 25 mm thick slice of bread (25 % penetration).

A comparison of loaf volumes for dough 6 and 7 showed that the addition of 1.5 mg of the variant increased the loaf volume by 9 %.

The results for firmness and elasticity are shown in Figs. 1 and 2. A comparison of doughs 6, 7 and 8 shows that the variant gives significantly softer crumb and significantly better elasticity from day 0 to day 7.

Example 10: DGDGase activity

The hydrolytic activity towards DGDG (di-galactosyl-di-glyceride) was determined by monolayer assay (DGDGase activity) as follows:

On a thoroughly purified surface of a buffer solution (10 mM NaOAc, pH 5.5; 1 mM CaCl2, 25°C; 10 mM beta-cyclodextrin (Sigma C-4767)) a monolayer of DGDG

(Sigma (D4651)) is spread from a chloroform solution. After relaxation of the monolayer (evaporation of chlorofom) the surface pressure is adjusted to 15 mN/m. A solution containing approximately 60 µg (micro gram) enzyme is injected through the monolayer into the subphase of the re-action compartment (cylinder with surface area 2230 mm^2 and reaction volume 56570 mm^3) in the "zero-order trough". Enzymatic activity is manifested through increased speed of a mobile barrier compressing the monolayer in order to maintain constant surface pressure as insoluble substrate molecules are hydrolysed into more water soluble reaction products (in presence of beta cyclodextrin).

The result is considered positive for DGDGase if the barrier moves at more than 1 mm/min. The results were as follows:

	Variant (see footnotes in Example 5)	DGDGase activity
Invention	*4)	Yes
	*2)	Yes
	*1)	Yes
	*3)	Yes
Reference	Lipolase	No
	FoL	Yes

CLAIMS

1. A variant of a parent lipolytic enzyme, which variant:

   a) comprises an alteration which is an insertion, a deletion or a substitution of an amino acid residue, at a position which is within 10 amino acid positions from the C-terminal, and

   b) has a higher phospholipase activity than the parent lipolytic enzyme.

2. A variant of a parent lipolytic enzyme having a lid and an alcohol binding site, which variant:

   a) comprises an alteration which is an insertion, a deletion or a substitution of an amino acid residue, at a position which is

      i) within 10 amino acid positions from the C-terminal, or

      ii) no more than 10 Å preferably no more than 8 Å) from the C atom at the sn2 position of the glycerol part of a substrate triglyceride, or

      iii) in the lipolytic enzyme lid, and

   b) has a higher phospholipase activity than the parent lipolytic enzyme.

3. The variant of either preceding claim which has a phospholipase activity greater than 0.1 nmol/min in a monolayer assay at pH 5 as described herein.

4. The variant of any preceding claim which has a phospholipase activity greater than 100 PHLU/mg preferably greater than 500 PHLU/mg) and/or a ratio of phospholipase activity to lipase activity greater than 0.1 PHLU/LU preferably greater than 0.5 PHLU/LU).

5. The variant of any preceding claim wherein the parent lipolytic enzyme has a phospholipase activity below 50 PHLU/mg and/or a ratio of phospholipase activity to lipase activity below 0.1 PHLU/LU.

6. The variant of any preceding claim which is native to an eukaryote, preferably to a fungus.

7. The variant of any preceding claim wherein the parent lipolytic enzyme belongs to the *Zygomycete* family.

8. The variant of any of claims 1-6 wherein the parent lipolytic enzyme belongs to the *Humicola* family.

9. The variant of the preceding claim wherein the parent lipolytic enzyme is the lipase of *Humicola lanuginosa* strain DSM 4109.

10. The variant of any preceding claim which comprises an alteration which is a substitution at a position corresponding to G266 in the *Humicola lanuginosa* lipase, preferably a substitution G266A, C, D, N, L, I, S, T, P or V.

11. The variant of any preceding claim which comprises an alteration at a position corresponding to position G263, L264, I265, T267 or L269 in the *Humicola lanuginosa* lipase, preferably a substitution which is G263A,E,Q,R; L264A,C,P,Q; I265L,N,T; T267A,Q or L269N.

12. The variant of any preceding claim which comprises an alteration in the lid which is a substitution of a negatively charged amino acid residue with a neutral or positively charged amino acid residue, or a substitution of a neutral amino acid residue with a positively charged amino residue.

13. The variant of the preceding claim which comprises an alteration in the lid at a position corresponding to position G91, D96 and/or E99 in the *Humicola lanuginosa* lipase, preferably a substitution which is G91A, D96S,W or E99K.

14. The variant of any of claims 8-10 wherein the alteration in the alcohol binding site comprises deletion of amino acid residues at positions corresponding to positions C268 and L269 in the lipase derived from *Humicola lanuginosa* strain DSM 4109.

15. The variant of any preceding claim which comprises a peptide extension at th C-terminal, preferably comprising 1-5 amino acid residues, the first preferably being A, P or D, the second if present) preferably being V, G or R, the third if present) preferably being V, G or R, the fourth if present) preferably being F, and the fifth if present) preferably being S.

16. A DNA sequence encoding the variant of any preceding claim.

17. A vector comprising the DNA sequence of the preceding claim.

18. A transformed host cell harboring the DNA sequence of claim 16 or the vector of claim 17.

19. A method of producing the variant of any of claims 1-15 comprising

a) cultivating the cell of claim 18 so as to express and preferably secrete the variant, and

b) recovering the variant.

20. A method of producing a phospholipase, which method comprises:

a) selecting a parent lipolytic enzyme,

b) identifying one or more amino acid residues in the parent lipolytic enzyme at positions which are within 10 amino acid positions from the C-terminal

c) making alterations each of which is an insertion, a deletion or a substitution of the amino acid residue,

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b),

e) preparing the variant resulting from steps b-d,

f) testing the phospholipase activity of the variant,

g) optionally repeating steps b-f,

h) selecting a variant having higher phospholipase activity than the parent lipolytic enzyme, and

i) producing the variant to obtain the phospholipase.

21. A method of producing a phospholipase, which method comprises:

a) selecting a parent lipolytic enzyme having a lid and an alcohol binding site,

b) identifying one or more amino acid residues in the parent lipolytic enzyme at positions which are:

i) within 10 amino acid positions from the C-terminal, or

ii) no more than 10 Å from the C atom at the sn2 position of the glycerol part of a substrate triglyceride, or

iii) in the lipolytic enzyme lid, and

c) making alterations each of which is an insertion, a deletion or a substitution of the amino acid residue,

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b),

e) preparing the variant resulting from steps b-d,

f) testing the phospholipase activity of the variant,

g) optionally repeating steps b-f,

h) selecting a variant having higher phospholipase activity than the parent lipolytic enzyme, and

i) producing the variant to obtain the phospholipase.

22. A process for preparing a dough or a baked product prepared from the dough which comprises adding the variant of any of claims 1-15 to the dough.

23. The process of the preceding claim which further comprises adding to the dough an endo-amylase and a phospholipid.

24. The process of either preceding claim wherein the endo-amylase is from *Bacillus*, and is preferably a maltogenic amylase from *B. stearothermophilus*.

25. A process for reducing the content of phospholipid in an edible oil, comprising treating the oil with the variant of any of claims 1-15 so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil.

Primers

4244: 5'-TCA AGA ATA GTT CAA ACA AGA AGA-3'

AP: 5'-GGT TGT CTA ACT CCT TCC TTT TCG-3'

FOL 14: 5'-TGT CCC YMG WCT CCC KCC K-3'

FOL 15: 5'-GAA GTA MYR YAG RTG MGC AGS RAT ATC-3'

FOL16: 5'-GAT ATY SCT GCK CAY CTR YRK TAC TTC-3'

H7: 5'-CGG AAT GTT AGG CTG GTT ATT GC-3'

KBoj 14: 5'-CTT TTC GGT TAG AGC GGA TG-3'

KBoj 32: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CTA 122 345 345 S67 C8A 91011 S1213 1.14.15 161718 T1920 C2122 GAA GTA CCA TAG GTG CGC AG23 GAT ATC CGG

KBoj 33: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CGC CGC GCA CTA C8A 91011 S1213 1.14.15 161718 T1920 C2122 GAA GTA CCA TAG GTG CGC AG23 GAT ATC CGG

KBoj34: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CTA 122 345 345 S67 201818 91011 S1213 1.14.15 161718 T1920 C2122 GAA GTA CCA TAG GTG CGC AG23 GAT ATC CGG

KBoj36: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CTA GTT ACA GGC GTC AGT CGC CTG GAA G

KBoj37: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CTA AGC GTT ACA GGC GTC AGT CGC CTG G

KBoj38: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CTA ACC AGC GTT ACA GGC GTC AGT CGC C

KBoj39: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CTA GCC ACC AGC GTT ACA GGC GTC AGT C

Distribution of nucleotides for each doped position

1: A 90, C 10

2: G 3,A 91,T 3,C 3

3: A 25, T 75

4: G 2, A 4, T 5, C 89

5: G 2, A 13, T 4, C 81

6: G 91, A 3, T 3, C 3

7: G 48, A 2, T 2, C 48

8: A 92, T8

9: A 97, T 3

10: G 1, A 1, T 1, C 97

11: G 1, A 97, T 1, C 1
12: G 94, A 2, T 2, C 2
13: G 1, A 1, T 91, C 7
14: G 1, A 1, T 7, C 91
15: G 2, A 2, T 2, C 94
16: A 80, T 20
17: G 6, A 90, T 2, C 2
18: G 2, A 2, T 94, C 2
19: G 5, A 91, T 4
20: G 96, C4
21: G 4, T 5, C 91
22: G 4, C 96
23: G 94, C 3, T 3

```
5559.020-DK
          1                                                        50
  rhimi   SIDGGIRAAT SQEINELTYY TTLSANSYCR TVIPGA...T WDC..IHCDA
  rhidl   SDGGKVVAAT TAQIQEFTKY AGIAATAYCR SVVPGN...K WDC..VQCQK
  SP400   ~~~~~~~EVS QDLFNQFNLF AQYSAAAYCG KNNDAPAGTN ITCTGNACPE
    Pcl   ~~~~~~~DVS TSELDQFEFW VQYAAASYYE ADYTAQVGDK LSCSKGNCPE
FoLnp11   ~~~~~~~GVT TTDFSNFKFY IQHGAAAYC. .NSEAAAGSK ITCSNNGCPT

          51                                                       100
  rhimi   TE..DLKIIK TWS.TLIYDT NAMVARGDSE KTIYIVFRGS SSIRNWIADL
  rhidl   WV.PDGKIIT TFT.SLLSDT NGYVLRSDKQ KTIYLVFRGT NSFRSAITDI
  SP400   VEKADATFLY SFEDSGVGDV TGFLALDNTN KLIVLSFRGS RSIENWIGNL
    Pcl   VEATGATVSY DFSDSTITDT AGYIAVDHTN SAVVLAFRGS YSVRNWVADA
FoLnp11   VQGNGATIVT SFVG.SKTGI GGYVATDSAR KEIVVSFRGS INIRNWLTNL

          101                                                      150
  rhimi   TFVPVSY.PP VSGTKVHKGF LDSYGEVQNE LVATVLDQFK QYPSYKVAVT
  rhidl   VFNFSDY.KP VKGAKVHAGF LSSYEQVVND YFPVVQEQLT AHPTYKVIVT
  SP400   NFDLKEINDI CSGCRGHDGF TSSWRSVADT LRQKVEDAVR EHPDYRVVFT
    Pcl   TFVHTNP.GL CDGCLAELGF WSSWKLVRDD IIKELKEVVA QNPNYELVVV
FoLnp11   DFGQEDC.SL VSGCGVHSGF QRAWNEISSQ ATAAVASARK ANPSFNVIST

          151                                                      200
  rhimi   GHSLGGATAL LCALDLYQRE EGLSSSNLFL YTQGQPRVGD PAFANYVVST
  rhidl   GHSLGGAQAL LAGMDLYQRE PRLSPKNLSI FTVGGPRVGN PTFAYYVEST
  SP400   GHSLGGALAT VAGADLRGN. .GY...DIDV FSYGAPRVGN RAFAEFLTVQ
    Pcl   GHSLGAAVAT LAATDLRGK. .GYP..SAKL YAYASPRVGN AALAKYITAQ
FoLnp11   GHSLGGAVAV LAAANLRVG. .GT...PVDI YTYGSPRVGN AQLSAFVSNQ

          201                                                      250
  rhimi   G.IPYRRTVN ERDIVPHLPP AAFGFLHAGE EYWITD.NSP ......ETVQ
  rhidl   G.IPFQRTVH KRDIVPHVPP QSFGFLHPGV ESWIKS.GT. ......SNVQ
  SP400   TGGTLYRITH TNDIVPRLPP REFGYSHSSP EYWIKS.GTL V.PVTRNDIV
    Pcl   G..NNFRFTH TNDPVPKLPL LSMGYVHVSP EYWITS.PNN A.TVSTSDIK
FoLnp11   A.GGEYRVTH ADDPVPRLPP LIFGYRHTTP EFWLSGGGGD KVDYTISDVK

          251                                                      300
  rhimi   VCTSDLETSD CSNSIVPFT. .SVLDHLSYF GINTGLCT~~ ~~~~~~~~~~
  rhidl   ICTSEIETKD CSNSIVPFT. .SILDHLSYF DINEGSCL~~ ~~~~~~~~~~
  SP400   KIEGID.ATG GNNQP.NIP. .DIPAHLWYF .GLIGTCL~~ ~~~~~~~~~~
    Pcl   VIDGDV.SFD GNTGTGLPLL TDFEAHIWYF .VQVDAGKGP GLPFKRV~~~
FoLnp11   VCEGAA.NLG CNGGT.LGL. .DIAAHLHYF .QATDACNAG GFSWRRYRSA

          301                                              338
  rhimi   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~...
  rhidl   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~...
  SP400   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~...
    Pcl   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~...
FoLnp11   ESVDKRATMT DAELEKKLNS YVQMDKEYVK NNQARS..
```

Fig. 1

Alignment of lipase sequences

PCT/DK99/00664
09/856819

DK 99/664

4

Kongeriget Danmark

Patent application No.: PA 1999 01481

Date of filing: 15 October 1999

Applicant: Novo Nordisk A/S
Novo Allé
DK-2880 Bagsværd

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Patent- og Varemærkestyrelsen
Erhvervsministeriet

TAASTRUP 17 December 1999

Karin Schlichting
Head Clerk

LIPOLYTIC ENZYME VARIANTS

FIELD OF THE INVENTION

The present invention relates to a method of altering the substrate specificity of a lipolytic enzyme by modifying the amino acid sequence, and to lipolytic enzyme variants obtained by such modification. The invention also relates to a DNA sequence encoding the variant, a vector comprising the DNA sequence, a transformed host cell harboring the DNA sequence or the vector, to a method of producing the variant, and to methods of using the variant.

BACKGROUND OF THE INVENTION

Lipolytic enzymes (such as lipases and phospholipases) are capable of hydrolyzing carboxylic ester bonds in a substrate to release carboxylic acids. The hydrolytic activity on different ester bonds is important for the usefulness of the lipolytic enzyme in various industrial applications.

Thus, enzymes with a high phospholipase activity are useful in a wide range of applications such as baking (US 4,567,046), filtration of wheat starch hydrolysate (US 5,264,367) and treatment of vegetable oil to reduce the content of phospholipid (US 5,264,367). For the treatment of vegetable oil, the enzyme should have a low lipase activity, i.e. a low hydrolytic activity towards ester bonds in triglycerides..

WO 98/45453 indicates that an enzyme with a high hydrolytic activity on digalactosyl diglyceride (DGDG) is useful in baking.

It is generally known to add a lipase to laundry detergents to aid in the removal of greasy soils (e.g. EP 258,068). EP 430,315 states that such laundry detergents may sometimes leave residual odors attached to the cloth, e.g. when soiled with a dairy product such as milk.

The release of short-chain fatty acids as free fatty acids (FFA) may be desirable for flavor development in food products, e.g. in cheese ripening (M. Hanson, ZFL, 41 (10), 664-666 (1990)).

The three-dimensional (3D) structure of several lipolytic enzymes is known, and several structures are known to contain a so-called "lid" which may be in an open or closed state covering the active site. Brady et al., Nature, 343, 767-770 (1990). Brzozowski A M et al., Nature, 351, 491 (1991). Derewenda et al., Biochemistry, 31 (5), 1532-1541 (1992).

F. Hara et al., JAOCS, 74 (9), 1129-32 (1997) indicates that some lipases have a certain phospholipase activity, whereas most lipases have little or no activity on phospholipids. Thus, phospholipase activity has been described in the lipases from

guinea pig pancreas, *Fusarium oxysporum* and *Staphylococcus hyicus*, and attempts have been made to relate the phospholipase activity to the structure of the lipase. WO 98/26057; M.D. van Kampen et al., Chemistry and Physics of Lipids, 93 (1998), 39-45; A. Hjorth et al., Biochemistry 1993, 32, 4702-4707.

The prior art has described the effect on chain-length selectivity by amino acid substitutions in a lipase from *Rhizopus delemar*. Thus, R. D. Joerger et al., Lipids, 29 (6), 377-384 (1994) indicates that the variants F95D, F112W and V209W have an altered preference to C_4 and C_8 acids. R. R. Klein et al., JAOCS, 74 (11), 1401-1407 (1997) shows that the variant V206T+F95D has a higher selectivity for C_8 acid. R. R. Klein et al., Lipids, 32 (2), 123-130 (1997) indicates that the variants V209W+F112W, V94W and F95D+F214R have a higher hydrolytic activity towards C_4 and C_8 acids, and suggests that structural determinants for medium-chain length specificity may reside in the distal end of the acyl binding groove.

SUMMARY OF THE INVENTION

The inventors have found that the substrate specificity of a lipolytic enzyme can be modified by making alterations to the amino acid sequence in a defined region of the lipolytic enzyme, so as to increase the level of a desired activity or to decrease the level of an undesired activity. Thus, the inventors have developed lipolytic enzymes with a modified amino acid sequence (hereinafter called lipolytic enzyme variants, or variants for short) with a substrate specificity which can be tailored for specific uses.

Accordingly, the invention provides a method of producing a lipolytic enzyme variant and lipolytic enzyme variants prepared by the method. The method comprises:

a) selecting a substrate and an ester bond of interest,

b) selecting a parent lipolytic enzyme,

c) selecting one or more amino acid residues in a region near the active site, near the C-terminal or in the lid region of the parent lipolytic enzyme as described below,

d) making alterations each of which is an insertion, a deletion or a substitution of the amino acid residue,

e) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than c),

f) preparing the resulting variant,

g) testing the activity of the variant on the ester bond in the substrate, and

h) selecting a variant having an altered activity on the ester bond.

Thus, in one aspect, the parent lipolytic enzyme has an alcohol binding site having a glycerol part with an sn2 position, and the amino acid alteration is within 10 Å of the C atom at the sn2 position of the glycerol part of a substrate triglyceride.

In another aspect, the parent lipolytic enzyme has a structure comprising a catalytic triad consisting of an active Ser, an active Asp and an active His residue, and the amino acid to be altered is either located between the active His residue of the catalytic residue and the C-terminal, or belongs to a set E defined by the following steps:

i) aligning the structure of the lipolytic enzyme with *Rhizomucor miehei* lipase structure 4TGL comprising a catalytic triad and an inhibitor phosphorus atom (4TGL-inhP), so as to minimize the sum of squares of deviation between atoms of the catalytic triads of the two structures,

ii) defining a set A consisting of atoms of the lipolytic enzyme inside a sphere of radius 18 Å with center at 4TGL-inhP,

iii) forming a first plane defined by 4TGL-inhP, the Cα atom of the active Ser residue of the parent lipolytic enzyme, and the Cα atom of the active Asp residue of the parent lipolytic enzyme and defining a set B as a subset of set A consisting of atoms on the same side of the first plane as the Cα atom of the active His residue of the parent lipolytic enzyme,

iv) forming a second plane defined by 4TGL-inhP, the Cα atom of the active Ser residue of the parent lipolytic enzyme, and the Cα atom of the active His residue of the parent lipolytic enzyme and defining a set C as a subset of set A consisting of atoms on the opposite side of the second plane from the Cα atom of the active Asp residue of the parent lipolytic enzyme,

v) forming a set D consisting of atoms belonging to the union of sets B and C, and having a solvent accessibility of 15 or higher, and

vi) forming set E consisting of amino acid residues in the structure which comprise an atom belonging to set D or an atom belonging to the union of sets B and C and located less than 3.5 Å from an atom belonging to set D,

In a third aspect, the lipolytic enzyme has an active site comprising an active His residue, and the alteration is made in the amino acid sequence between the active His residue and the C-terminal.

In yet another aspect of the invention, the amino acid alteration is made among the 10 amino acid residues at the C-terminal.

In a further aspect, the parent lipolytic enzyme has a lid, and the alteration is made in the lid.

The invention also provides a DNA sequence encoding the variant, an expression vector comprising the DNA sequence, a transform d host cell harboring the DNA

sequence or the expression vector, and to a method of producing the variant by cultivating the transformed host cell so as to produce the variant and recovering th variant from the resulting broth. Further, the invention provides uses of the variants.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows an alignment of lipase sequences.

DETAILED DESCRIPTION OF THE INVENTION

Altered activity on selected ester bond in substrate

Compared to the parent lipolytic enzyme, the invention aims to alter the activity on a selected ester bond in a substrate, i.e. either to increase a desired activity or decrease an undesired activity.

Thus, an enzyme with increased phospholipase activity may be useful, e.g., in baking or in purification of vegetable oil. It may be desired to increase the hydrolytic activity on digalactosyl-diglyceride (DGDG) for use in baking. It may be desired to increase the lipase activity on long-chain (C_{16}-C_{20}) triglycerides for any industrial use where lipases are used or the lipase activity on short-chain or medium-chain (C_4-C_8) triglycerides for use in flavor development in food products (such as cheese ripening).

Conversely, it may be desired to decrease the lipase activity on long-chain (C_{16}-C_{20}) triglycerides for use as a phospholipase in purification of vegetable oil. It may be desired to decrease the lipase activity on short-chain or medium-chain (C_4-C_8) for use in baking or in detergents.

Parent lipolytic Enzyme

The lipolytic enzyme to be used in the present invention is one that can hydrolyze ester bonds. Such enzymes include, for example, lipases, such as triacylglycerol lipase (EC 3.1.1.3), lipoprotein lipase (EC 3.1.1.34), monoglyceride lipase (EC 3.1.1.23), lysophospholipase, ferulic acid esterase and esterase (EC 3.1.1.1, EC 3.1.1.2). The numbers in parentheses are the systematic numbers assigned by the Enzyme Commission of the International Union of Biochemistry in accordance with the type of the enzymatic reactivity of the enzyme.

The parent lipolytic enzyme may be prokaryotic, particularly a bacterial enzyme, e.g. from *Pseudomonas*. Examples are *Pseudomonas* lipas s, .g. from *P. cepacia* (US 5,290,694, pdb file 1OIL), *P. glumae* (N Frenken t al. (1992), Appl. Envir.

Microbiol. 58 3787-3791, pdb files 1TAH and 1QGE), *P. pseudoalcaligenes* (EP 334 462) and *Pseudomonas sp.* strain SD 705 (FERM BP-4772) (WO 95/06720, EP 721 981, WO 96/27002, EP 812 910). The *P. glumae* lipase sequence is identical to the amino acid sequence of *Chromobacterium viscosum* (DE 3908131 A1). Other examples are bacterial cutinases, e.g. from *Pseudomonas* such as *P. mendocina* (US 5,389,536) or *P. putida* (WO 88/09367).

Alternatively, the parent lipolytic enzyme may be eukaryotic, e.g. a fungal lipolytic enzyme such as lipolytic enzymes of the *Humicola* family and the *Zygomycetes* family and fungal cutinases.

Examples of fungal cutinases are the cutinases of *Fusarium solani pisi* (S. Longhi et al., Journal of Molecular Biology, 268 (4), 779-799 (1997)) and *Humicola insolens* (US 5,827,719).

The *Humicola* family of lipolytic enzymes consists of the lipase from *H. lanuginosa* strain DSM 4109 and lipases having more than 50 % homology with said lipase. The lipase from *H. lanuginosa* (synonym *Thermomyces lanuginosus*) is also referred to as Lipolase®. It is described in EP 258 068 and EP 305 216, and has the amino acid sequence shown in positions 1-269 of SEQ ID NO: 2 of US 5,869,438.

The *Humicola* family also includes the following lipolytic enzymes: lipase from *Penicillium camembertii* (P25234), lipase/phospholipase from *Fusarium oxysporum* (EP 130064, WO 98/26057), lipase from *F. heterosporum* (R87979), lysophospholipase from *Aspergillus foetidus* (W33009), phospholipase A1 from *A. oryzae* (JP-A 10-155493), lipase from *A. oryzae* (D85895), lipase/ferulic acid esterase from *A. niger* (Y09330), lipase/ferulic acid esterase from *A. tubingensis* (Y09331), lipase from *A. tubingensis* (WO 98/45453), lysophospholipase from *A. niger* (WO 98/31790).

The *Zygomycetes* family comprises lipases having at least 50 % homology with the lipase of *Rhizomucor miehei* (P19515). This family also includes the lipases from *Absidia reflexa*, *A. sporophora*, *A. corymbifera*, *A. blakesleeana*, *A. griseola* (all described in WO 96/13578 and WO 97/27276) and *Rhizopus oryzae* (P21811). Numbers in parentheses indicate publication or accession to the EMBL, GenBank, GeneSeqp or Swiss-Prot databases.

It is of particular interest to derive a variant with phospholipase activity from a parent lipolytic enzyme having no or very little phospholipase activity, e.g. corresponding to a ratio of phospholipase activity to lipase activity below 0.1 PHLU/LU or below 50 PHLU/mg.

Alteration near alcohol binding site

As already stated, the amino acid sequence of the parent lipolytic enzyme may be modified at a position which near the glycerol part of a substrate triglyceride. This

region will be referred to as the "alcohol binding site" of the lipase; it is described in Brzozowski A M et al., Nature, 351: 491 (1991); Uppenberg et al., Biochemistry, 1995, 34, 16838-16851; A. Svendsen, Inform, 5(5), 619-623 (1994).

For the *Rhizomucor miehei* lipase, the extent of the alcohol binding site can be found from the PDB file "5tgl.pdb" available in Structural Classification of Proteins (SCOP) on the Internet, at http://www.rcsb.org/pdb/, showing the complex with the inhibitor n-hexylphosphonate ethyl ester which mimics the substrate. It is described in Derewenda et al. (supra), Brzozowski et al. (supra) and Brady et al. (supra). The sn2 position of this model is the atom CE2.

The variant typically contains no more than 10 alterations in the alcohol binding site, e.g. 1, 2, 3, 4, 5 or 6 alterations.

The alteration may particularly be in that part of the alcohol binding site which comes within 20 positions (e.g. within 10 positions) of the C-terminal.

As already stated, the amino acid sequence of the parent lipolytic enzyme may be modified at a position which is within 10 Å (e.g. within 8 Å, particularly within 6 Å) of the C atom at the sn2 position of the glycerol part of a substrate triglyceride. The following amino acid positions lie within 10 Å of the sn2 position in the *Rhizomucor miehei* lipase: 25, 28, 80-84, 88, 143-146, 175, 203, 205, 254-255, 257-259, 264-267. The following are within 8 Å: 81-83, 144, 257-258, 265-267, and the following within 6 Å: 82, 144, 257, 266.

In the *Humicola lanuginosa* lipase, the following positions are within 10 Å of the sn2 position: 18, 21, 81-85, 89, 145-148, 172, 201, 203, 255-256, 258-260, 264-267. The following are within 8 Å: 82-84, 89, 146, 258-259, 265-267, and the following within 6 Å: 83, 146, 258, 266.

Alteration near catalytic triad

As already stated, in one aspect the parent lipolytic enzyme has a structure comprising a catalytic triad consisting of an active Ser, an active Asp and an active His residue, and the amino acid to be altered belongs to a set defined by a certain procedure described above. The structure may be an open or a closed structure, and it may or may not include a substrate or an inhibitor.

The procedure is conveniently performed by use of software such as MSI's Insight II. It involves alignment with 4TGL, a crystal structure of the lipase from *Rhizomucor miehei* inhibited irreversibly by diethyl p-nitrophenyl phosphate. This is available in Structural Classification of Proteins (SCOP) on the Internet, at http://www.rcsb.org/pdb/, and is described in Derewenda et al. (supra). The *Rhizomucor miehei* lipase comprises a catalytic triad consisting of the amino acid residues S144, D203 and H 257.

For the *Humicola lanuginosa* lipase, the structure 1tib may be used; it is available in Structural Classification of Prot ins (SCOP) on the Internet. Using this structure, the set defined by the procedure includes the following positions: 10-23, 26, 40, 55-64, 80-87, 116-117, 119, 145-149, 151, 168, 170, 194, 196-201, 220-222, 224-227, and 254-269.

To prepare variants of a lipolytic enzyme of the *Humicola* family, the amino acid alterations may preferably be made at positions corresponding to 20-25, 56-64, 81-85 or 255-269 in the *Humicola lanuginosa* lipase. Thus, the alteration may be a substitution, deletion or insertion at a position corresponding to A20, Y21, G23, K24, N25, V63, R81, G82, R84, A257, W260, Y261, F262 or G266 (preferably excluding G23C, K24C, R81C), a substitution of an amino acid corresponding to C268 or L269, a substitution corresponding to V60G, D62E, P256A, G263E,Q,R,F, L264A,C,P,F,G,I, I265L,N,F or T267A,Q,P,S,E, or an insertion corresponding to T267GS or T267GL.

To alter the activity towards short-chain (C_4-C_8) fatty acids in triglycerides, alterations may be made at positions corresponding to Y21, E56, D57, V60, G61, D62, R81, S83, R84, L259, Y261 or G266. To increase the activity for DGDG, alterations may be made at positions corresponding to Y21, G23, N26, D57, D62, R81, S83, R84, S85, G266, T267 or L269; preferably, two or more such alterations are made, preferably together with one or more alterations in the lid region. To increase the phospholipase activity, alterations may be made at positions corresponding to R81, R84, S85, or 263-267, e.g. G266 or T267.

To prepare variants of a *Pseudomonas* lipase, amino acid modifications are preferably made at positions corresponding to 12-13, 16-34, 45-52, 59-66, 68, 86-87, 107-109, 111, 143-153, 155, 157-158, 207-212, 228, 230, 242-249, 264, 279-280, 282-297, 301-302, 304-305, 307-308 in the *P. cepacia* lipase, particularly L17/L17, T18/A18, Y29/Y29, L287/L286, E289/E288, I290/I289, Q292/Q291 or L293/L292 in the *P. cepacia*/*P. glumae* lipase.

Alteration between active His residue and C-terminal

As stated above, one or more alterations may be made in the amino acid sequence between an active His residue and the terminal, preferably among the 12 amino acids at the C-terminal side of the active His.

The *Humicola lanuginosa* lipase has an active His at H258 and the C-terminal at L269, so this region includes positions 259-269. The *P. cepacia* lipase has an active H286 and the C-terminal at residue 297, so the region includes residues 287-297.

Alterati n near C-terminal

As stated above, one or more alterations may b mad within 10 amino acid positions from the C-terminal of the mature protein,

or at positions corresponding to such positions in the *H. lanuginosa* lipase, i.e. positions 260-269 of the *H. lanuginosa* lipase. Corresponding positions may be found by alignment of the two sequences as described later in this specification.

The lipolytic enzyme variant may be truncated by deleting amino acid residues corresponding to the first 1, 2, 3, 4, 5 or 6 positions at the C-terminal. A truncated variant may have improved thermostability.

Alternatively, the variant may carry a peptide extension at the C-terminal and/or the N-terminal. The C-terminal extension may consist of 1-10 amino acid residues, e.g. A, P, AG, DG, PG, AGG, PVGF, AGRF, PRGF, AGGF or AGGFS; or it may consist of 40-50 residues, e.g., consisting of the 48 C-terminal residues of the *Fusarium oxysporum* lipase AGGFSWRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS. The C-terminal extension may increase the phospholipase activity.

Some alterations in the region overlapping with the alcohol binding site are described below.

A preferred alteration is a substitution at a position corresponding to G266 in the *Humicola lanuginosa* lipase, preferably with an amino acid of intermediate size, e.g. A, C, D, N, L, I, S, T, P or V. Such alteration alone has been found sufficient to increase the phospholipase activity.

Other preferred alterations are such that alter the tertiary structure, e.g. by introducing bulky side chains or by disrupting the bond angles, e.g. by introducing Pro. Such alterations may be made at positions corresponding to positions G263, L264, I265, T267 or L269 in the *Humicola lanuginosa* lipase. Some preferred substitutions are G263A,E,Q,R; L264A,C,P,Q; I265L,N,T; T267A,Q or L269N.

Alteration in lid

As stated above, the amino acid sequence of the prent lipolytic enzyme may be modified in the lid region of the parent lipolytic enzyme. This region is described in Brady et al., Nature 343, 1990, pp. 767-770 and in Brzozowski A M et al., Nature, 351: 491 (1991). In the *H. lanuginosa* lipase, the lid is located at positions 80-100, and the modification may particularly be made at positions 82-98, e.g. 91-98.

The variant typically contains no more than 5 alterations in the lid region; it may contain 0, 1, 2 or 3 alterations. A preferred alteration is a substitution of an amino acid corresponding to G91, L93, N94, D96, K98, L97 and/or E99 in the *Humicola lanuginosa* lipase with a neutral or positively charged amino acid, e.g. a substitution corresponding to G91A,T, L93K, N94D, D96S,W,G, L97Q, K98D,F,E and/or E99K,D.

Preferably, a variant with an alteration in the lid region also contains one or more alterations near the catalytic triad, near the substrate binding site or near the C-terminal.

Lipolytic enzyme variants

The lipolytic enzyme variant of the invention comprises an alteration (i.e., an insertion, a deletion or a substitution of an amino acid residue) in the regions described above. The total number of alterations in the above regions is typically not more than 20, e.g. not more than 10 or not more than 5, and there may be as little as 1 or 2 alterations in the above regions.

In addition, the lipolytic enzyme variant of the invention may optionally include other modifications of the parent enzyme, typically not more than 10, e.g. not more than 5 such modifications.

The variant generally has a homology with the parent lipolytic enzyme of at least 90 %, typically at least 95 %.

The variant of the invention may further comprise a peptide extension at the N-terminal, preferably consisting of 1-15 (particularly 4-10) amino acid residues, and preferably comprising 1, 2 or 3 positively charged amino acids. Some preferred N-terminal peptide extensions are AS, SPIRR, E1RP, E1SPIRPRP, E1SPPRRP and E1SPIRPRP. Further, any peptide extension described in WO 97/04079 and WO 97/07202 may be used.

Homology and alignment

For purposes of the present invention, the degree of homology may be suitably determined by means of computer programs known in the art, such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-45), using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

In the present invention, corresponding (or homologous) positions in the lipase sequences of *Rhizomucor miehei* (rhimi), *Rhizopus delemar* (rhidl), *Thermomyces lanuginosa* (former; *Humicola lanuginosa*) (SP400), *Penicillium camembertii* (Pcl) and *Fusarium oxysporum* (FoLnp11), are defined by the alignment shown in Figure 1.

To find the homologous positions in lipase sequences not shown in the alignment, the sequence of interest is aligned to the sequences shown in Figure 1. The new sequence is aligned to the present alignment in Fig. 1 by using the GAP alignment to the most homologous sequence found by the GAP program. GAP is provided in the GCG program package (Program Manual for the Wisconsin Packag , Version 8,

August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-45). The following settings are used for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

Preferred variants

Preferred variants of the *H. lanuginosa* lipase are those disclosed in the examples and the two variants G266R and G266K. Corresponding alterations may be made in other parent lipolytic enzymes. Further variants may be derived from these by omitting amino acid modifications at positions 1, 106, 186, 225, 232, 237, 239 or 274. Variants with 274S may optionally have a further C-terminal extension of WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS (corresponding to the C-terminal of the *F. oxysporum* lipase) in full or truncated form.

Nomenclature for amino acid alterations

The nomenclature used herein for defining mutations is basically as described in WO 92/05249. Thus, G91A indicates substitution of G in position 91 with A. T267A,Q indicates substitution of T at position 267 with A or Q. E1E,D,A indicates that E1 is unchanged or is substituted with D or A.

T267stop indicates a stop codon, i.e. deletion of T267 and all following amino acids (i.e. C268 and L269). 270P, 271V indicates a C-terminal extension of PV (i.e. at new positions 270 and 271). -G266 indicates deletion of G at position 266. Parentheses indicate that the alteration is optional, or in examples that the alteration is uncertain. SPIRR indicates an N-terminal extension. D266 may refer to the position or to substitution with any amino acid (except D).

E1SPPCGRRP indicates a substitution of E1 with SPPCGRRP, i.e. a peptide addition at the N-terminal. T267GS indicates a substitution of T267 with GS, or in other words the substitution T267G and an insertion of S between G267 and C268.

Phospholipase activity

As described above, the variant of the invention may have a higher phospholipase activity than the parent lipolytic enzyme. By the monolayer method described later in this specification, the variant preferably has a phospholipase activity of at least 0.1 nmol/min at pH 5.

By the PHLU method described later in this specification, the variant preferably has a phospholipase activity of at least 100 PHLU/mg (mg of pure enzyme protein), particularly at least 500 PHLU/mg. The variant has a ratio of phospholipase activity to

lipase activity (both measured at pH 7) of at least 0.1 PHLU/LU, preferably at least 0.5, particularly at least 2.

The variants of the invention may have the ability to hydrolyze intact phospholipid, as demonstrated by the PHLU method. They may have A_1 and/or A_2 activity, so they may be able to hydrolyze one or both fatty acyl groups in the phospholipid.

pH optimum

Many Lipolase variants of the invention have an alkaline pH optimum for lipase activity and an acid pH optimum for phospholipase activity (e.g. pH 9-10 for lipase and pH 4-6 for phospholipase). Such variants can be used at acid pH (e.g. in oil degumming, described later), as phospholipases with very low concomitant lipase activity.

However, some Lipolase variants which include the substitution G266D,E have pH optima for both lipase and phospholipase activities around pH 5-6. Such variants may be used at acid pH when both lipase and phospholipase activities are desired, e.g. in baking.

Thermostability

The thermostability of the variant can conveniently be evaluated by means of Differential Scanning Calorimetry (DSC). Depending on exact mutations, the variants of the invention generally have similar or slightly lower thermostability than the parent lipolytic enzyme.

The temperature at the top of the denaturation peak (T_d) of the lipase from *Humicola lanuginosa* when heated at 90 deg/hr at pH 5 is just above 70 °C (=T_d). T_d for the variants of the invention is generally 5-10 degrees lower

Use of variant

Depending on the substrate specificity, variants of the invention can be used, e.g., in filtration improvement, vegetable oil treatment, baking, detergents, or preparation of lysophospholipid.

Improvement of filtration

A variant with lysophospholipase activity can be used to improve the filterability of an aqueous solution or slurry of carbohydrate origin by treating it with the variant. This is particularly applicable to a solution or slurry containing a starch hydrolysate, especially a wheat starch hydrolysate since this tends to be difficult to filter and to give cloudy filtrates. The treatment can be done in analogy with EP 219,269 (CPC International).

Veg table oil tr atment

A variant with phospholipase activity can be used in a process for reducing the content of phospholipid in an edible oil, comprising treating the oil with the variant so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil. This process is applicable to the purification of any edible oil which contains phospholipid, e.g. vegetable oil such as soy bean oil, rape seed oil and sunflower oil. The treatment is preferably carried out at acid pH, e.g. pH 3-5. Advantageously, a variant can be selected so as to have a high phospholipase activity and a low lipase activity at low pH, due to different pH optima of the two activities.

The process for oil treatment can be conducted according to principles known in the art, e.g. in analogy with US 5,264,367 (Metallgesellschaft, Röhm); K. Dahlke & H. Buchold, INFORM, 6 (12), 1284-91 (1995); H. Buchold, Fat Sci. Technol., 95 (8), 300-304 (1993); JP-A 2-153997 (Showa Sangyo); or EP 654,527 (Metallgesellschaft, Röhm).

Miscellaneous uses of phospholipase

A variant with phospholipase activity can be used to prepare lysophospholipid (e.g. lyso-lecithin) by treating the corresponding phospholipid with the variant, e.g. as described in EP 870840, JP-A 10-42884, JP-A 4-135456 or JP-A 2-49593. The variant can also be used to make mayonnaise, e.g. as described in EP 628256, EP 398666 or EP 319064.

A variant with phospholipase activity may also be used in the processing of dairy and other food products, e.g. as described in EP 567,662 (Nestlé), EP 426,211 (Unilever), EP 166,284 (Nestlé), JP-A 57-189638 (Yakult) or US 4,119,564 (Unilever).

A variant with activity towards short-chain fatty acyl groups may be used to release free fatty acids (FFA) for flavor development in food products, e.g. in cheese ripening, e.g. as described in M. Hanson, ZFL, 41 (10), 664-666 (1990)).

The variant may be used leather treatment, as described in JP-A 7-177884 (Kao).

Baking

A variant with phospholipase activity can be used in the preparation of dough, bread and cakes, e.g. to increase dough stability and dough handling properties, or to improve the elasticity of the bread or cake. Thus, the variant can be used in a process for making bread, comprising adding the variant to the ingredients of a dough, kneading the dough and baking the dough to make the bread. This can be done in analogy

with US 4,567,046 (Kyowa Hakko), JP-A 60-78529 (QP Corp.), JP-A 62-111629 (QP Corp.), JP-A 63-258528 (QP Corp.) or EP 426211 (Unilever).

It is particularly advantageous to use the variant together with an anti-staling endo-amylase and optionally also to add a phospholipid, to reduce-staling of the bread and particularly to improve softness of the bread in the first 24 hours after baking. The endo-amylase may be a maltogenic α-amylase (e.g. from *Bacillus sp.*, such as Novamyl® from Novo Nordisk) or a fungal or bacterial α-amylase, e.g. from *Aspergillus* or *Bacillus*, particularly *A. oryzae*, *B. licheniformis* or *B. amyloliquefaciens*.

In baking, it may be preferred to use a variant having a low activity on short-chain or medium-chain (C_4-C_8) for use in baking or in detergents. The use of such a variant may avoid or suppress the development of an undesired flavor due to the release of short-chain fatty acids.

Use in detergent

The variant may be used as a detergent additive, preferably at a concentration (expressed as pure enzyme protein) of 0.001-10 (e.g. 0.01-1) mg per gram of detergent or 0.001-100 (e.g. 0.01-10) mg per liter of wash liquor.

In detergents, it is preferred to use a variant with high activity on long-chain triglycerides (C_{16}-C_{20}) to improve the removal of fatty soiling. It may also be preferred to use a variant with phospholipase activity.

It is preferred to use a variant with low activity towards short-chain (C_4-C_8) fatty acids in triglycerides. The use of such a variant may avoid or suppress the development of an undesired odor due to the release of short-chain fatty acids.

Variants having both lipase and phospholipase activity at alkaline pH may be used in detergents.

Detergent composition

The detergent composition of the invention may for example be formulated as a hand or machine laundry detergent composition including a laundry additive composition suitable for pre-treatment of stained fabrics and a rinse added fabric softener composition, or be formulated as a detergent composition for use in general household hard surface cleaning operations. In a laundry detergent, the variant may be effective for the removal of fatty stains, for whiteness maintenance and for dingy cleanup. A laundry detergent composition may be formulated as described in WO 97/04079, WO 97/07202, WO 97/41212, PCT/DK WO 98/08939 and WO 97/43375.

The detergent composition of the invention may particularly be formulated for hand or machine dishwashing operations. e.g. as described in GB 2,247,025 (Unilever) or WO 99/01531 (Procter & Gamble). In a dishwashing composition, the variant may be

effective for removal of greasy/oily stains, for prevention of the staining /discoloration of the dishware and plastic components of the dishwasher by highly colored components and the avoidance of lime soap deposits on the dishware.

The detergent composition of the invention may be in any convenient form, e.g., a bar, a tablet, a powder, a granule, a paste or a liquid. A liquid detergent may be aqueous, typically containing up to 70 % water and 0-30 % organic solvent, or non-aqueous.

The detergent composition comprises one or more surfactants, which may be non-ionic including semi-polar and/or anionic and/or cationic and/or zwitterionic. The surfactants are typically present at a level of from 0.1% to 60% by weight, e.g. 0.5-40 %, preferably 1-30 %, typically 1.5-20 %.

When included therein the detergent will usually contain from about 1% to about 40% of an anionic surfactant such as linear alkylbenzenesulfonate, alpha-olefinsulfonate, alkyl sulfate (fatty alcohol sulfate), alcohol ethoxysulfate, secondary alkanesulfonate, alpha-sulfo fatty acid methyl ester, alkyl- or alkenylsuccinic acid or soap.

When included therein the detergent will usually contain from about 0.2% to about 40% of a non-ionic surfactant such as alcohol ethoxylate, nonyl-phenol ethoxylate, alkylpolyglycoside, alkyldimethylamine-oxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, polyhydroxy alkyl fatty acid amide, or N-acyl N-alkyl derivatives of glucosamine ("glucamides").

The invention also provides a detergent additive comprising the variant of the invention. The detergent additive as well as the detergent composition may comprise one or more other enzymes such as a protease, a lipase, a cutinase, an amylase, a carbohydrase, a cellulase, a pectinase, a mannanase, an arabinase, a galactanase, a xylanase, an oxidase, e.g., a laccase, and/or a peroxidase.

In general the properties of the chosen enzyme(s) should be compatible with the selected detergent, (i.e. pH-optimum, compatibility with other enzymatic and non-enzymatic ingredients, etc.), and the enzyme(s) should be present in effective amounts.

Proteases: Suitable proteases include those of animal, vegetable or microbial origin. Microbial origin is preferred. Chemically modified or protein engineered mutants are included. The protease may be a serine protease or a metallo protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from Bacillus, e.g., subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270 and WO 94/25583.

Examples of useful proteases are the variants described in WO 92/19729, WO 98/20115, WO 98/20116, and WO 98/34946, especially the variants with substitutions in one or more of the following positions: 27, 36, 57, 76, 87, 97, 101, 104, 120, 123, 167, 170, 194, 206, 218, 222, 224, 235 and 274.

Preferred commercially available protease enzymes include Alcalase ®, Savinase ®, Primase ®, Duralase ®, Esperase ®, and Kannase ® (Novo Nordisk A/S), Maxatase ®, Maxacal ®, Maxapem ®, Properase ®, Purafect ®, Purafect OxP ®, FN2 ™, and FN3 ™ (Genencor International Inc.).

Lipases: Suitable lipases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful lipases include lipases from *Humicola* (synonym *Thermomyces*), e.g. from *H. lanuginosa* (*T. lanuginosus*) as described in EP 258 068 and EP 305 216 or from *H. insolens* as described in WO 96/13580, a *Pseudomonas* lipase, e.g. from *P. alcaligenes* or *P. pseudoalcaligenes* (EP 218 272), *P. cepacia* (EP 331 376), *P. stutzeri* (GB 1,372,034), *P. fluorescens*, *Pseudomonas sp.* strain SD 705 (WO 95/06720 and WO 96/27002), *P. wisconsinensis* (WO 96/12012), a *Bacillus* lipase, e.g. from *B. subtilis* (Dartois et al. (1993), Biochemica et Biophysica Acta, 1131, 253-360), *B. stearothermophilus* (JP 64/744992) or *B. pumilus* (WO 91/16422).

Other examples are lipase variants such as those described in WO 92/05249, WO 94/01541, EP 407 225, EP 260 105, WO 95/35381, WO 96/00292, WO 95/30744, WO 94/25578, WO 95/14783, WO 95/22615, WO 97/04079 and WO 97/07202.

Preferred commercially available lipase enzymes include Lipolase ™ and Lipolase Ultra ™ (Novo Nordisk A/S).

Cellulases: Suitable cellulases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Suitable cellulases include cellulases from the genera *Bacillus, Pseudomonas, Humicola, Fusarium, Thielavia, Acremonium*, e.g. the fungal cellulases produced from Humicola insolens, *Myceliophthora thermophila* and *Fusarium oxysporum* disclosed in US 4,435,307, US 5,648,263, US 5,691,178, US 5,776,757 and WO 89/09259.

Especially suitable cellulases are the alkaline or neutral cellulases having colour care benefits. Examples of such cellulases are cellulases described in EP 0 495 257, EP 0 531 372, WO 96/11262, WO 96/29397, WO 98/08940. Other examples are cellulase variants such as those described in WO 94/07998, EP 0 531 315, US 5,457,046, US 5,686,593, US 5,763,254, WO 95/24471, WO 98/12307 and PCT/DK98/00299.

Commercially available cellulases include Celluzyme ®, and Carezyme ® (Novo Nordisk A/S), Clazinase , and Puradax HA (Genencor International Inc.), and KAC-500(B) (Kao Corporation).

Peroxidases/Oxidases: Suitable per-oxidases/oxidases include those of plant, bac-terial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful peroxidases include peroxidases from *Coprinus*, e.g. from *C. cinereus*, and variants thereof as those described in WO 93/24618, WO 95/10602, and WO 98/15257.

Commercially available peroxidases include Guardzyme® (Novo Nordisk A/S).

The detergent enzyme(s) may be included in a detergent composition by adding separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes. A detergent additive of the invention, i.e. a separate additive or a combined additive, can be formulated e.g. as a granulate, a liquid, a slurry, etc. Preferred detergent additive formulations are granulates, in particular non-dusting granulates, liquids, in particular stabilized liquids, or slurries.

Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molar weights of 1000 to 20000; ethoxylated nonyl-phenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in GB 1483591. Liquid enzyme preparations may, for in-stance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent may contain 0-65 % of a detergent builder or complexing agent such as zeolite, diphosphate, tripho-sphate, phosphonate, carbonate, citrate, nitrilotriacetic acid, ethylenediaminetetraacetic acid, diethylenetri-aminepen-taacetic acid, alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst).

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose, poly(vinyl-pyrrolidone), poly (ethylene glycol), poly(vinyl alcohol), poly(vinylpyridine-N-oxide), poly(vinylimidazole), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid co-polymers.

The detergent may contain a bleaching system which may comprise a H2O2 source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine or nonanoyloxybenzenesul-fonate. Alternatively, the bleaching system may comprise peroxyacids of e.g. the amide, imide, or sulfone type.

The enzyme(s) of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g., a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative, e.g., an aromatic borate ester, or a phenyl boronic acid derivative such as 4-formylphenyl boronic acid, and the composition may be formulated as described in e.g. WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as e.g. fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil redeposition agents, dyes, bactericides, optical brighteners, hydrotropes, tarnish inhibitors, or perfumes.

It is at present contemplated that in the detergent compositions any enzyme, in particular the variant of the invention, may be added in an amount corresponding to 0.01-100 mg of enzyme protein per liter of wash liquor, preferably 0.05-5 mg of enzyme protein per liter of wash liquor, in particular 0.1-1 mg of enzyme protein per liter of wash liquor.

The variant of the invention may additionally be incorporated in the detergent formulations disclosed in WO 97/07202 which is hereby incorporated as reference.

Methods for preparing enzyme variants

The enzyme variant of the invention can be prepared by methods known in the art, e.g. as described in WO 97/04079 (Novo Nordisk). The following describes methods for the cloning of enzyme-encoding DNA sequences, followed by methods for generating mutations at specific sites within the enzyme-encoding sequence.

Cloning a DNA sequence encoding a enzyme

The DNA sequence encoding a parent enzyme may be isolated from any cell or microorganism producing the enzyme in question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the enzyme to be studied. Then, if the amino acid sequence of the enzyme is known, labeled oligonucleotide probes may be synthesized and used to identify enzyme-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labeled oligonucleotide probe containing sequences homologous to another known enzyme gene could be used as a probe to identify enzyme-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying enzyme-encoding clones would involve inserting fragments of genomic DNA into an expression v ctor, such as a plasmid, transforming enzyme-negative bacteria with the resulting genomic DNA library, and

then plating the transformed bacteria onto agar containing a substrate for enzyme (*i.e.* maltose), thereby allowing clones expressing the enzyme to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, *e.g.* the phosphoroamidite method described S.L. Beaucage and M.H. Caruthers, (1981), Tetrahedron Letters 22, p. 1859-1869, or the method described by Matthes et al., (1984), EMBO J. 3, p. 801-805. In the phosphoroamidite method, oligonucleotides are synthesized, *e.g.* in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al., (1988), Science 239, 1988, pp. 487-491.

Site-directed mutagenesis

Once a enzyme-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites. In a specific method, a single-stranded gap of DNA, the enzyme-encoding sequence, is created in a vector carrying the enzyme gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al., (1984), Biotechnology 2, p. 646-639. US 4,760,025 discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method for introducing mutations into enzyme-encoding DNA sequences is described in Nelson and Long, (1989), Analytical Biochemistry 180, p. 147-151. It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Further, Sierks. et al., (1989) "Site-directed mutagenesis at the activ site Trp120 of *Aspergillus awamori* glucoamylase. Protein Eng., 2, 621-625; Sierks et al., (1990), "Catalytic mechanism of fungal glucoamylase as defined by mutagenesis of Asp176, Glu179 and Glu180 in the enzyme from *Aspergillus awamori*". Protein Eng. vol. 3, 193-198; also describes site-directed mutagenesis in an *Aspergillus* glucoamylase.

Expression of enzyme variants

According to the invention, a DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

Expression vector

The recombinant expression vector carrying the DNA sequence encoding a enzyme variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. The vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated. Examples of suitable expression vectors include pMT838.

Promoter

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the DNA sequence encoding a enzyme variant of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dag*A promoters, the promoters of the *Bacillus licheniformis* α-amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* α-amylase (*amyQ*), the promoters of the *Bacillus subtilis* xylA and xylB genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, the TPI (triose phosphate isomerase) promoter from *S. cerevisiae* (Alber et al. (1982), J. Mol. Appl. Genet 1, p. 419-434, *Rhizomucor miehei* aspartic proteinase, *A. niger* neu-

tral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

Expression vector

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the α-amylase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, *e.g.* as described in WO 91/17243.

The procedures used to ligate the DNA construct of the invention encoding a enzyme variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989).

Host Cells

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of a enzyme variant of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, *e.g.* by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may b a c ll of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are Gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gramnegative bacteria such as *E.coli*. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The yeast organism may favorably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, *e.g. Saccharomyces cerevisiae*.

The host cell may also be a filamentous fungus *e.g.* a strain belonging to a species of *Aspergillus*, most preferably *Aspergillus oryzae* or *Aspergillus niger, or* a strain of *Fusarium*, such as a strain of *Fusarium oxysporium*, *Fusarium graminearum* (in the perfect state named *Gribberella zeae,* previously *Sphaeria zeae, synonym with Gibberella roseum and Gibberella roseum* f. sp. *cerealis)*, or *Fusarium sulphureum (*in the prefect state named *Gibberella puricaris,* synonym with *Fusarium trichothecioides, Fusarium bactridioides, Fusarium sambucium, Fusarium roseum,* and *Fusarium roseum var. graminearum), Fusarium cerealis (*synonym with *Fusarium crokkwellnse), or Fusarium venenatum.*

In a preferred embodiment of the invention the host cell is a protease deficient of protease minus strain.

This may for instance be the protease deficient strain *Aspergillus oryzae* JaL 125 having the alkaline protease gene named "alp" deleted. This strain is described in WO 97/35956 (Novo Nordisk).

Filamentous fungi cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. The use of *Aspergillus* as a host micro-organism is described in EP 238 023 (Novo Nordisk A/S), the contents of which are hereby incorporated by reference.

Method of producing the enzyme variant of the invention

The enzyme variant of the invention may be produced by a method comprising cultivating a host cell under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the nzyme vari-

ant of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (*e.g.* as described in catalogues of the American Type Culture Collection).

The enzyme variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Expression of variant in plants

The present invention also relates to a transgenic plant, plant part or plant cell which has been transformed with a DNA sequence encoding the variant of the invention so as to express and produce this enzyme in recoverable quantities. The enzyme may be recovered from the plant or plant part. Alternatively, the plant or plant part containing the recombinant enzyme may be used as such.

The transgenic plant can be dicotyledonous or monocotyledonous, for short a dicot or a monocot. Examples of monocot plants are grasses, such as meadow grass (blue grass, Poa), forage grass such as festuca, lolium, temperate grass, such as Agrostis, and cereals, e.g. wheat, oats, rye, barley, rice, sorghum and maize (corn).

Examples of dicot plants are tobacco, legumes, such as lupins, potato, sugar beet, pea, bean and soybean, and cruciferous (family Brassicaceae), such as cauliflower, oil seed rape and the closely related model organism Arabidopsis thaliana.

Examples of plant parts are stem, callus, leaves, root, fruits, seeds, and tubers. In the present context, also specific plant tissues, such as chloroplast, apoplast, mitochondria, vacuole, peroxisomes and cytoplasm are considered to be a plant part. Furthermore, any plant cell, whatever the tissue origin, is considered to be a plant part.

Also included within the scope of the invention are the progeny of such plants, plant parts and plant cells.

The transgenic plant or plant cell expressing the variant of the invention may be constructed in accordance with methods known in the art. In short the plant or plant cell is constructed by incorporating one or more expression constructs encoding the variant of the invention into the plant host genome and propagating the resulting modified plant or plant cell into a transgenic plant or plant cell.

Conveniently, the expression construct is a DNA construct which comprises a gene encoding the variant of the invention in operable association with appropriate regulatory sequences required for expression of the gene in the plant or plant part of choice. Furthermore, the expression construct may comprise a selectable marker useful

for identifying host cells into which the expression construct has been integrated and DNA sequences necessary for introduction of the construct into the plant in question (the latter depends on the DNA introduction method to be used).

The choice of regulatory sequences, such as promoter and terminator sequences and optionally signal or transit sequences is determined, eg on the basis of when, where and how the enzyme is desired to be expressed. For instance, the expression of the gene encoding the variant of the invention may be constitutive or inducible, or may be developmental, stage or tissue specific, and the gene product may be targeted to a specific tissue or plant part such as seeds or leaves. Regulatory sequences are eg described by Tague et al, Plant, Phys., 86, 506, 1988.

For constitutive expression the 35S-CaMV promoter may be used (Franck et al., 1980. Cell 21: 285-294). Organ-specific promoters may eg be a promoter from storage sink tissues such as seeds, potato tubers, and fruits (Edwards & Coruzzi, 1990. Annu. Rev. Genet. 24: 275-303), or from metabolic sink tissues such as meristems (Ito et al., 1994. Plant Mol. Biol. 24: 863-878), a seed specific promoter such as the glutelin, prolamin, globulin or albumin promoter from rice (Wu et al., Plant and Cell Physiology Vol. 39, No. 8 pp. 885-889 (1998)), a *Vicia faba* promoter from the legumin B4 and the unknown seed protein gene from *Vicia faba* described by Conrad U. et al, Journal of Plant Physiology Vol. 152, No. 6 pp. 708-711 (1998), a promoter from a seed oil body protein (Chen et al., Plant and cell physiology vol. 39, No. 9 pp. 935-941 (1998), the storage protein napA promoter from Brassica napus, or any other seed specific promoter known in the art, eg as described in WO 91/14772. Furthermore, the promoter may be a leaf specific promoter such as the rbcs promoter from rice or tomato (Kyozuka et al., Plant Physiology Vol. 102, No. 3 pp. 991-1000 (1993), the chlorella virus adenine methyltransferase gene promoter (Mitra, A. and Higgins, DW, Plant Molecular Biology Vol. 26, No. 1 pp. 85-93 (1994), or the aldP gene promoter from rice (Kagaya et al., Molecular and General Genetics Vol. 248, No. 6 pp. 668-674 (1995), or a wound inducible promoter such as the potato pin2 promoter (Xu et al, Plant Molecular Biology Vol. 22, No. 4 pp. 573-588 (1993).

A promoter enhancer element may be used to achieve higher expression of the enzyme in the plant. For instance, the promoter enhancer element may be an intron which is placed between the promoter and the nucleotide sequence encoding the enzyme. For instance, Xu et al. *op cit* disclose the use of the first intron of the rice actin 1 gene to enhance expression.

The selectable marker gene and any other parts of the expression construct may be chosen from those available in the art.

The DNA construct is incorporated into the plant genome according to conventional techniques known in the art, including *Agrobacterium*-mediated transformation,

virus-mediated transformation, micro injection, particle bombardment, biolistic transformation, and electroporation (Gasser et al, Science, 244, 1293; Potrykus, Bio/Techn. 8, 535, 1990; Shimamoto et al, Nature, 338, 274, 1989).

Presently, *Agrobacterium tumefaciens* mediated gene transfer is the method of choice for generating transgenic dicots (for review Hooykas & Schilperoort, 1992. Plant Mol. Biol. 19: 15-38), however it can also be used for transforming monocots, although other transformation methods are generally preferred for these plants. Presently, the method of choice for generating transgenic monocots is particle bombardment (microscopic gold or tungsten particles coated with the transforming DNA) of embryonic calli or developing embryos (Christou, 1992. Plant J. 2: 275-281; Shimamoto, 1994. Curr. Opin. Biotechnol. 5: 158-162; Vasil et al., 1992. Bio/Technology 10: 667-674). An alternative method for transformation of monocots is based on protoplast transformation as described by Omirulleh S, et al., Plant Molecular biology Vol. 21, No. 3 pp. 415-428 (1993).

Following transformation, the transformants having incorporated the expression construct are selected and regenerated into whole plants according to methods well-known in the art.

MATERIALS AND METHODS

Lipase activity on tributyrin (LU)

A substrate for lipase is prepared by emulsifying tributyrin (glycerin tributyrate) using gum Arabic as emulsifier. The hydrolysis of tributyrin at 30 °C at pH 7 is followed in a pH-stat titration experiment. One unit of lipase activity (1 LU) equals the amount of enzyme capable of releasing 1 µmol butyric acid/min at the standard conditions.

Lipase activity on triolein (SLU)

The lipolytic activity may be determined using olive oil as substrate.

In this SLU method, the lipase activity is measured at 30°C and pH 9 with a stabilized olive oil emulsion (Sigma catalog No. 800-1) as the substrate, in a 5 mM Tris buffer containing 40 mM NaCl and 5 mM calcium chloride. 2.5 ml of the substrate is mixed with 12.5 ml buffer, the pH is adjusted to 9, 0.5 ml of diluted lipase sample is added, and the amount of oleic acid formed is followed by titration with a pH stat.

One SLU is the amount of lipase which liberates 1 µmole of titratable oleic acid per minute under these conditions.

Phosph lipase activity

The following assay methods w re used for qualitative or quantitative d termination of phospholipase activity.

Phospholipase activity (PHLU)

Phospholipase activity (PHLU) is measured as the release of free fatty acids from lecithin. 50 µl 4% L-alpha-phosphatidylcholine (plant lecithin from Avanti), 4 % Triton X-100, 5 mM $CaCl_2$ in 50 mM HEPES, pH 7 is added 50 µl enzyme solution diluted to an appropriate concentration in 50 mM HEPES, pH 7. The samples are incubated for 10 min at 30 °C and the reaction stopped at 95 °C for 5 min prior to centrifugation (5 min at 7000 rpm). Free fatty acids are determined using the NEFA C kit from Wako Chemicals GmbH; 25 µl reaction mixture is added 250 µl Reagent A and incubated 10 min at 37 °C. Then 500 µl Reagent B is added and the sample is incubated again, 10 min at 37 °C. The absorption at 550 nm is measured using an HP 8452A diode array spectrophotometer. Samples are run in at least in duplicates. Substrate and enzyme blinds (preheated enzyme samples (10 min at 95 °C) + substrate) are included. Oleic acid is used as a fatty acid standard. 1 PHLU equals the amount of enzyme capable of releasing 1 µmol of free fatty acid/min at these conditions.

Phospholipase activity (LEU)

Lecithin is hydrolyzed under constant pH and temperature, and the phospholipase activity is determined as the rate of titrant (0.1N NaOH) consumption during neutralization of the liberated fatty acid.

The substrate is soy lecithin (L-α-Phosphotidyl-Choline), and the conditions are pH 8.00, 40.0°C, reaction time 2 min. The unit is defined relative to a standard.

Phospholipase monolayer assay

On a thoroughly purified surface of a buffer solution (either 10 mM Glycin, pH 9.0 or 10 mM NaOAc, pH 5.0; 1 mM CaCl2, 25°C) a monolayer of Di-Decanoyl-Phosphatidyl Choline (DDPC) is spread from a chloroform solution. After relaxation of the monolayer (evaporation of chlorofom) the surface pressure is adjusted to 15 mN/m, corresponding to a mean molecular area of DDPC of approx. 63 $Å^2$/molec. A solution containing approximately 60 µg (micro gram) enzyme is injected through the monolayer into the subphase of the re-action compartment (cylinder with surface area 2230 mm2 and reaction volume 56570 mm3) in the "zero-order trough". Enzymatic activity is manifested through the speed of a mobile barrier compressing the monolayer in order to maintain constant surface pressure as insoluble substrate molecules are hydrolyzed into more water soluble reaction products. Having verified that the aqueous solubility of the reaction products (capric acid and MDPC) are considerably higher than for DDPC

the number of DDPC-molecules hydrolyz d per minute by the enzyme is estimated from the mean molecular area (MMA) of DDPC. The results are calculated on basis of average barrier speed over the first 5 minutes of hydrolysis.

Plate assay 1

A) 50 ml 2% agarose in purified water is melted/stirred for 5 minutes and cooled to 60 - 63°C.

B) 50 ml 2% plant L-alpha-Phosphatidylcholine 95% in 0,2M NaOAc, 10 mM $CaCl_2$, pH 5,5 at 60°C in 30 min. is blended in 15 sec. with ultrathorax.

Equal volumes of 2% agarose and 2% Lecithin (A and B) are mixed, and an equal volume of 1 % Triton X-100 is added to this mixture. 250 µl 4 mg/ml crystal violet in purified water is added as indicator. The mixture is poured into appropriate petri dishes (e.g. 30 ml in 14cm Ø dish), and appropriate holes are made in the agar (3-5 mm) for application of enzyme solution.

The enzyme sample is diluted to a concentration corresponding to OD_{280} = 0.5 and 10 microliter is applied into holes in the agarose/lecithin-matrix. Plates are incubated at 30°C and reaction zones in the plates are identified after approx. 4-5 hours and/or after approx. 20 hours incubation. Lipolase is used as a control, and the presence of a larger clearing zone than the control is taken as a positive result for phospholipase activity.

In a variation of this assay, the addition of Triton X-100 is omitted.

Plate assay 2

10 g agarose is melted in 550 ml H2O by boiling in a microwave oven. After cooling to 60-70°C the following ingredients are added:

250 ml of a 0.4 M Citrate buffer (pH 4.5 or pH 7.1)

200 ml 3% lecithin (from Avanti) in 2% Triton-X 100

2 ml 2% crystal violet

30 ml of the mixture is poured into 14 cm Ø petri dishes.

The plates are incubated after application of enzyme samples, and the results are interpreted as for Plate assay 1.

MATERIALS AND METHODS

Yeast Strain

Saccharomyces cerevisiae YNG318: MATa leu2-D2 ura3-52 his4-539 pep4-D1[cir+] , described in WO 97/04079 and WO 97/07205.

Transf rmati n f yeast strain

Th DNA fragments and the opened vectors are mixed and transformed into the yeast *Saccharomyces cerevisiae* YNG318 by standard methods.

Vector for yeast transformation

pJSO026 (*S. cerevisiae* expression plasmid) is described in WO 97/07205 and in J.S.Okkels, (1996) "A URA3-promoter deletion in a pYES vector increases the expression level of a fungal lipase in Saccharomyces cerevisiae. Recombinant DNA Biotechnology III: The Integration of Biological and Engineering Sciences, vol. 782 of the Annals of the New York Academy of Sciences). It is derived from pYES 2.0 by replacing the inducible GAL1-promoter of pYES 2.0 with the constitutively expressed TPI (triose phosphate isomerase)-promoter from *Saccharomyces cerevisiae* (Albert and Karwasaki, (1982), J. Mol. Appl Genet., 1, 419-434), and deleting a part of the URA3 promoter.

Site-directed mutagenesis

For the construction of variants of a H. lanuginosa lipolytic enzyme the commercial kit, Chameleon double-stranded, site-directed mutagenesis kit can be used according to the manufacturer's instructions.

The gene encoding the lipolytic enzyme in question is inserted into the plasmid pHD414. In accord-ance with the manufacturer's instructions the ScaI site of the Ampicillin gene of pHD414 is changed to a MluI site by use of the following primer:

Primer 3: AGAAATCGGGTATCCTTTCAG.

The pHD414 vector comprising the lipolytic gene in question is then used as a template for DNA polymerase and oligos 7258 and 7770.

7258: 5'p gaa tga ctt ggt tga cgc gtc acc agt cac 3'

(Thus changing the ScaI site found in the ampicillin resistance gene and used for cutting to a MluI site).

Primer no. 7770 was used as the selection primer.

7770: 5'p tct agc cca gaa tac tgg atc aaa tc 3' (Changes the ScaI site found in the H. lanuginosa lipase gene without changing the amino acid sequence).

The desired mutation (e.g. in the N-terminal of the lipolytic gene or the introduction of a cystein residue) is introduced into the lipolytic gene in question by addition of an appropriate oligos comprising the desired mutation.

PCR reactions are performed according to the manufacturer's recomendations.

Screening meth d

The y ast libraries are spread on cellulose filters on SC-ura agar plates and incubated for 3-4 days at 30°C.

The filters are then transferred to the lecithin plates and incubated at 37°C for 2-6 hours. Yeast cells harbouring active phospholipases will develope white clearing zones around the colonies. The positive variants can then be further purified and tested.

Media

SC-ura medium

Yeast Nitrogen (without amino aicds)	7.5 g
Succinic acid	11.3 g
NaOH	6.8 g
Casaminoacid (without vitamins)	5.6 g
Tryptophan	0.1 g
Agar, Merck	20 g
Distilled water	ad 1000 ml

Autoclaved for 20 minutes at 121°C.

From a sterile stock solution of 5% Threonine 4 ml is added to a volume of 900 ml together with 100 ml of a sterile 20% glucose.

EXAMPLES

Example 1: Construction of variants with Lipolase "backbone" and C-terminal from *Fusarium oxysporum* phospholipase by PCR reaction

For the Lipolase backbone the following variants were used as templates: E1A +G91A +D96W +E99K +Q249R and SPIRR +G91A +D96W +E99K +Q249R. Wild-type Lipolase was used for generating a fragment in the C-terminal without Q249R. The template for the C-terminal phospholipase was the F.o. phospholipase, cloned in the same vector as the Lipolase variants.

PCR reaction 1: 4244 (SEQ ID NO: 1) as 5' primer and H7 (SEQ ID NO: 6) as 3'primer and one of the two templates mentioned above.

PCR reaction 2: FOL14 (SEQ ID NO: 3) as 5' primer and FOL15 (SEQ ID NO: 4) as 3' primer and wild-type Lipolase as template (no mutation in pos 249)

PCR reaction 3: FOL16 (SEQ ID NO: 5) as 5' primer and AP (SEQ ID NO: 2) as 3' primer and F.o. phospholipase as template

A PCR reaction 4 was made to create the connection between the Lipolase variant and the C-terminal from the phospholipase by using FOL14 (SEQ ID NO: 3) as 5' primer and AP (SEQ ID NO: 2) as 3' primer and PCR reaction 2 and 3 as template.

The final PCR was made with 4244 (SEQ ID NO: 1) as 5' primer and KBoj14 (SEQ ID NO: 7) as 3' primer and PCR re-action 1 and 4 as template. (By using wild-type Lipolase as template in reaction 2 a possibility to omit the mutation in position 249 was created).

The final PCR fragment was used in an in vivo recombination in yeast together with pJSO026 cut with th restriction enzymes. SmaI(or BamHI) and XbaI (to remove the coding region and at the same time create an overlap of about 75 bp in each end to make a recombination event possible). This final treatment was also used in the following examples.

Primer FOL14 (SEQ ID NO: 3) and primer 15/16 are mixed oligoes to give the possibility to bind both with Lipolase and phospholipase templates and at the same time give possibilities for introducing the amino acids from both templates in the different positions. For some of the positions new amino acids could be introduced as well.

Primer FOL14 (SEQ ID NO: 3)

Position 205 in the *H. lanuginosa* lipase: 75% R, 25% S

Primer FOL15 (SEQ ID NO: 4) /FOL16 (SEQ ID NO: 5)

Position 256 in the *H. lanuginosa* lipase: 50% P, 50% A

Position 260 in the *H. lanuginosa* lipase: 25% R, 12.5% Q, 12.5% H, 12.5% C, 12.5% Y, 12.5% W, 12.5% stop.

The sequences of the resulting variants were determined, and were found to correspond to Lipolase with the following alterations. Alterations in parentheses are uncertain.

E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

E1A, G91A, D96W, E99K, E239C, Q249R, P256A, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G273F, (274S)

E1A, G91A, D96W, E99K, N248T, Q249R, W260Q, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

SPIRR, G91A, D96W, E99K, W260C, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272, G273F, (274S)

SPIRR, G91A, D96W, E99K, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

E1A, G91A, D96W, E99K, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

Example 2: Production of truncated sequences

Variants were made with stop after amino acid 269, 270, 271, 272,(273 and 274)

The following PCR reactions were made with the following template: E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S).

Reaction 1: 5' primer 4244 (SEQ ID NO: 1) and 3' primer KBoj36 (stop after 269)

Reaction 2: 5' primer 4244 (SEQ ID NO: 1) and 3' primer KBoj37 (stop after 270)

Reaction 3: 5' primer 4244 (SEQ ID NO: 1) and 3' primer KBoj38 (stop after 271)

Reaction 4: 5' primer 4244 (SEQ ID NO: 1) and 3' primer KBoj39 (stop after 272)

The sequences of the resulting variants were determined, and were found to correspond to Lipolase with the following alterations:

E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N

E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A

E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G

E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G

Example 3: Removal of mutations in the lid region

G91A or E99K can be removed without loosing the phospholipase activity. The sequences of the resulting variants were determined, and were found to correspond to Lipolase with the following alterations:

E1A, G91A, D96W, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

SPIRR, D96W, E99K, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

SPIRR, G91A, D96W, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

E1A, G91A, D96W, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

Example 4: Doping in the C-terminal region of Lipolase to introduce phospholipase activity

Three different libraries were constructed with possibilities for mutations in position 256 and position 263-269. At the same time possibilities for extension of the C-terminal with either 1, 2 , 3 or 4 amino acids were included.

Doping, the wt sequences are underlined:

256: P 94, A 3, T 3

263: G 87, E 4.8, A 3.8, R 3.6, Q 0.2, P 0.2

264: L 87, P 4.8, Q 3.8, V 3.6, A 0.2, E 0.2

265: I 85, T 5.6, L 2.2, S 1.6, N 1.5, F 1.4, R 0.4, K 0.4 A,P 0.1, G,D,C,H,Y 0.03, Q,E 0.01, stop 0.016

266: G 86, D 5.9, R 2, S 1.7, C 1.6, A 0.9, V 0.9, E 0.7, W 0.2, H,Y 0.1, I,L,T,F,P 0.02, Q,K 0.01, stop 0.014

267: T 86, A 6.6, S 1.9, R 0.9, N 0.9, I 0.9, K 0.9, M 0.9, P 0.9, P 0.9, G,V 0.14, D,E 0.07, L 0.03, C,Q,H,F,W,Y 0.01, stop 0.01

268: C 91, S 1.9, R 1.0, G 1.0, F 0.9, Y 0.9, L 0.04, A,N,D,H,I,P,T,V 0.01, stop 2.8

269: L 92, stop 8 (KBoj 32 (SEQ ID NO: 8) and KBoj33)/ N 86, K 2.7, D 1.8, H 1.8, I 1.8, S 1.8, T 1.9, Y 1.8, R 0.1, Q,M,E 0.06, A,C,G,L,F,P,V 0.04, stop 0.06(KBoj34)

270: stop 100 (KBoj33)/A 44, P 44, S 1.9, T 1.8, R 1.5, L 1.5, G 1.4, V 1.4, D 0.7, Q 0.7, E 0.7, H 0.7, N,C,I,K,M,F,W,Y 0.03, stop 0.03 (KBoj 32 (SEQ ID NO: 8) and KBoj 34)

271: G 72, R 4.5, V 3.2, E 3.0, C 2.9, A 1.6, S 1.2, D 1.0, L 0.5, I,K,Y 0.15, Q,T 0.08, N,P 0.05, stop 9.2

272: G 72, R 4.5, V 3.2, E 3.0, C 2.9, A 1.6, S 1.2, D 1.0, L 0.5, I,K,Y 0.15, Q,T 0.08, N,P 0.05, stop 9.2

273: F 74, L 11, S 2.8, I 2.7, V 2.7, Y 2.5, C 2.5, A,R,T 0.1, N,D,H 0.08, Q,E,K 0.01, stop 0.5

274 STOP

Library A: PCR reaction with 4244 (SEQ ID NO: 1) as 5' primer and KBoj 33 as 3' primer and E1A +G91A +D96W +E99K +Q249R or E1A +G225R as template. Variants from this library will be without extension.

Library B: PCR reaction with 4244 (SEQ ID NO: 1) as 5' primer and KBoj 32 (SEQ ID NO: 8) as 3' primer and E1A +G91A +D96W +E99K +Q249R or E1A +G225R as template. Variants from this library will most probably contain a C-terminal extension but can contain stop codons before the extension.

Library C: PCR reaction with 4244 (SEQ ID NO: 1) as 5' primer and KBoj 34 as 3' primer and E1A +G91A +D96W +E99K +Q249R or E1A +G225R as template. Variants from this library will most probably contain mutations in position 269 and a C-terminal extension but can contain stop codons before the extension.

The following variants were obtained:

Library A:

E1A +G91A +D96W +E99K +Q249R +G266D

Library B:

E1A +G91A +D96W +E99K +(R232L) +Q249R +G266S +270A

E1A +G91A +D96W +E99K +Q249R +G266S +270D +271G

E1A+ G91A+ D96W+ E99K+ Q249R+ L264G+ I265G+ G266F+ T267stop

E1A +G91A +D96W +E99K +Q249R +G266A +270P +271G

E1A +G91A +D96W +E99K +Q249R +L264P +I265F +L269stop

Library C:

E1A +G91A +D96W +E99K +Q249R +G263E +G266D +L269N +270P +271V +272G +273F

E1A +G91A +D96W +E99K +Q249R +G263A +G266S +L269N +270A +271G +272R +273F

E1A +G91A +D96W +E99K +Q249R +L264P -G266 +L269I +270P +271R +272G +273F

E1A +G91A +D96W +E99K +Q249R +G266D +L269S +270A +271G +272G +273F

E1A +D27G +G91A +D96W +E99K +Q249R +G266S +L269N +270A +271G +272G +273F

E1A +G91A +D96W +E99K +Q249R +G266D +L269N +270A

E1A +G91A +D96W +E99K +Q249R +L264P +L267Q +L269N

E1A +G91A +D96W +E99K +Q249R +G263R +I265L +L269N +270P

Example 5: Lipase and phospholipase activities of variants

The phospholipase activity of variants was measured by the PHLU assay. The following variants of Lipolase were found to have phospholipase activity.

1) E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

2) SPIRR, G91A, D96W, E99K, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

3) E1A, G91A, D96W, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

4) E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N

5) E1A, G91A, D96W, E99K, Q249R, G266S, 270D, 271G

6) E1A, G91A, D96W, E99K, Q249R, G266D

7) E1A, G91A, D96W, E99K, Q249R, G266A, 270P, 271G

8) G266D

Several of the variants had a higher ratio of phospholipase (PHLU) to lipas (LU) than a prior-art enzyme from *F. oxysporum* known to have both lipase and phospholipase activity.

Example 6: pH optimum of lipase and phospholipase activity

For some of the above variants, the pH optimum for lipase and phospholipase was determined by using the LU and PHLU methods at various pH values. The results showed that the pH optimum phospholipase activity was in the range 4-6. The optimum for lipase activity varied from about pH 6 to about pH 10.

Example 7: Phospholipase activity by monolayer assay

The same 8 variants as in Example 5 were analyzed for phospholipase activity by the mono layer assay described above at pH 5 and 9. The results showed all variants have phospholipase activity at pH 5 and 9, whereas the parent lipase (Lipolase) showed no activity at pH 5 or 9. Depending on the variant, the activity at pH 5 was higher or lower than at pH 9.

A prior-art variant of Lipolase was found to have no phospholipase activity at pH 5: SPIRR +N94K +F95L +D96H +N101S +F181L +D234Y +I252L +P256T +G263A +L264Q.

Example 8: Phospholipase activity of variants

The following variants of Lipolase were found to have phospholipase activity by one of the assay methods described above. The parent enzyme (Lipolase) did not show this activity.

E1SPPCGRRP +E99N +E239C +Q249R +G266D
E1SPPCGRRP +E239C +Q249R +G266D
E1SPPCGRRP +L93K +E99K +E239C +Q249R +G266D
E1SPPCGRRP +E99K +E239C +Q249R +G266D
G266A
G266W
G266V
G263Q +L264A +I265T +G266D +T267A
G263F +L264A +G266S +T267E
E1SPPCGRRP +E239C +Q249R +G263Q +L264A +I265T +G266D +T267A
G266S

G266L
G263A +G266A
G263A +G266Y
E1SPPCGRRP +E239C +Q249R +G266A
E1SPPCGRRP +E239C +Q249R +G266S
E1SPPCGRRP +E239C +Q249R +G263F +L264A +G266S +T267E
D62A + G266A
D62A + G266S
D96S + G266A
D96S+ G266S
D96S+ G266R
D96S+ G266W
D96S+ G266V
E1SPPCGRRP + G91A+ D96W+ E239C+ Q249R+ G266D
E1SPPCGRRP + G91A+ D96W+ E239C+ Q249R+ G266S
E1SPPCGRRP + G91A+ D96W+ E239C+ Q249R+ G263E+ G266S+ 270A
E1SPPCGRRP + G91A+ D96W+ E239C+ Q249R+ L264P+ G266S
E1SPPCGRRP + G91A+ D96W+ E239C+ Q249R+ P256T+ G266D
E1SPPCGRRP + G91A+ D96W+ E239C+ Q249R+ G266C+ T267P+ L269stop
G263D +L264I +I265N +G266E +T267GS
E219G +L264I +I265N +G266T +T267GL
E1A+ G91A+ D96W+ E99K+ P256A+ W260H+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S)
E1A+ G91A+ D96W+ E99K+ E239C+ Q249R+ P256A+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G +273F (+274S)
E1A+ G91A+ D96W+ E99K+ N248T+ Q249R+ W260Q+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S)
SPIRR+ G91A+ D96W+ E99K+ W260C+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272+ G273F (+274S)
SPIRR+ G91A+ D96W+ E99K+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S)
E1A+ G91A+ D96W+ E99K+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S)
E1A+ G91A+ D96W+ E99K+ P256A+ W260H+ G263Q+ L264A+ I265T+ G266D+

T267A+ L269N+ 270A+ 271G +272G +273F (+274S)
SPIRR+ D96W+ E99K+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S)
SPIRR+ G91A+ D96W+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S)
E1A+ G91A+ D96W+ E99K+ P256A+ W260H+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N
E1A+ G91A+ D96W+ E99K+ Q249R+ G263E+ G266D+ L269N+ 270P+ 271V+ 272G+ 273F
E1A+ G91A+ D96W+ E99K+ Q249R+ G263A+ G266S+ L269N+ 270A+ 271G+ 272R+ 273F
E1A+ G91A+ D96W+ E99K+ Q249R+ L264P+ Δ266+ L269I+ 270P+ 271R+ 272G+ 273F
E1A+ G91A+ D96W+ E99K+ Q249R+ L264C+ I265N+ G266P+ T267stop
E1A+ G91A+ D96W+ E99K (+R232L)+ Q249R+ G266S+ 270A
E1A+ G91A+ D96W+ E99K+ Q249R+ G266S+ 270D+ 271G
E1A+ G91A+ D96W+ E99K+ Q249R+ L264F+ Δ266+ 270A+ 271G+ 272G+ 273F
E1A+ G91A+ D96W+ E99K+ Q249R+ L264G+ I265G+ G266F+ T267stop
E1A+ G91A+ D96W+ E99K+ Q249R+ L264stop
E1A+ G91A+ D96W+ E99K+ P256A+ W260H+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G
E1A+ G91A+ D96W+ E99K+ P256A+ W260H+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G
E1A+ G91A+ D96W+ E99K+ Q249R+ G266D
E1A+ G91A+ D96W+ E99K+ Q249R+ G266D
E1A+ G91A+ D96W+ E99K+ Q249R+ G266A+ 270P+ 271G
E1A+ G91A+ D96W+ E99K+ Q249R+ L264P+ I265F+ L269stop
E1A+ G91A+ D96W+ E99K+ Q249R+ G266D+ L269S+ 270A+ 271G+ 272G+ 273F
E1A+ G91A+ D96W+ E99K+ Q249R+ G266D+ L269N+ 270A
E1A+ G91A+ D96W+ E99K+ Q249R+ G266S+ L269N+ 270A+ 271G+ 272G+ 273F
E1A+ G91A+ D96W+ E99K+ Q249R+ L264P+ L267Q+ L269N
E1A+ G91A+ D96W+ E99K+ Q249R+ G263R+ I265L+ L269N+ 270P
E1A+ D96W+ E99K+ P256A+ W260H+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S)
E1A+ G225R+ G266D

E1A+ G225R+ G263A+ I265V+ G266S
E1A+ G225R+ G263A+ T267A
E1SPPCGRRP+ D96S+ E239C+ Q249R+ I252M+ L264Q+ G266D
E1SPPCGRRP+ G91A+ D96W+ E239C+ Q249R+ G266D
E1SPPCGRRP+ D96S+ E239C+ Q249R+ G266D
E1SPPCGRRP+ D96S+ E239C+ Q249R+ G266C+ L267A
E1A+ G91A+ D96W+ E99K+ Q249R+ G266A
E1A+ D96M+ G106S+ G225R+ G266D
E1A+ D96Q+ G106S+ G225R+ G266S
E1A+ D96F+ G225R+ G266S
E1A+ D96C+ G225R+ G266T
E1A+ D96H+ G106S+ G225R+ G266S
SPIRR+ D96S+ G266D
SPIRR+ D96R+ G106S+ G266D
SPIRR+ D96I+ G106S+ G266S
SPIRR+ D96W+ K237R+ G266S
SPIRR+ G266A
SPIRR+ D96S+ G106S+ G225R+ G266D
SPIRR+ D96Q+ G106S+ G225R+ G266A
SPIRR+ D96Y+ G106S+ G225R+ G266N
SPIRR+ D96C+ G106S+ G225R+ G266T
SPIRR+ D96H+ T186I+ G225R+ G266S
E1SPPRRP+ G91A+ D96W+ E239C+ Q249R+ G266D
E1SPPRRP+ G91A+ D96W+ E239C+ Q249R+ G266S
E1SPPRRP+ G91A+ D96W+ E239C+ Q249R+ G263E+ G266S+ 270A
E1SPPRRP+ G91A+ D96W+ E239C+ Q249R+ L264P+ G266S
E1SPPRRP+ G91A+ D96W+ E239C+ Q249R+ P256T+ G266D
E1SPPRRP+ G91A+ D96W+ E239C+ Q249R+ G266C+ T267P+ L269stop
E1A+ G91A+ D96W+ E99K+ Q249R+ G266S+ T267S
E1SPPCGRRP+ G91A+ D96W+ E239C+ Q249R+ P256T+ G266S
E1SPPCGRRP+ E239C+ Q249R+ P256T+ G266S+ T267A
E1SPPCGRRP+ E239C+ Q249R+ G266D

E1SPPCGRRP+ G91A+ D96W+ E239C+ Q249R+ G266D
E1SPPRRP+ D96S+ E239C+ Q249R+ G266D
L259S
G266D
G91A +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A+ 271G+ 272G+ 273F (+274S)
G266E
G263A +G266A
E1SPCRPRP +E239C +Q249R +G266A
E1SPCRPRP +E239C +Q249R +G266S
D96S + G266A
D96S + G266S
D96S + G266W
E1SPPCGRRP +D96S +E239C +Q249R +G263D +L264I +I265N +G266E +T267GS
E1SPPCGRRP +D96S +E239C +Q249R +L264I +I265N +G266T +T267GL
D96F +G266A
D96F +G266S
E1SPPCGRRP +E99N +E239C +Q249R +G266A
E1SPPCGRRP + D96S +E239C +Q249R +G266A
E1SPPCGRRP + D96S +E239C +Q249R +G266S
E1SPPCGRRP + D96S +E239C +Q249R +G263F +L264A +G266S +T267E
V60G +D62A +S83T +R84K +D96W +G266D
V60G +D62A +S83T +D96W +G266D
V60G +D62A +S83T +D96W +G266W
L259I
L259N
D96W +G263Q +L264A +I265T +G266D +T267A

In the table above, (+274S) indicates that the presence of this amino acid residue at the C-terminal is uncertain. For one such variant, it was found that only a minor fraction contained this residue

Example 9: Chain-length specificity of variants

A number of Lipolase variants were tested for their hydrolytic activity on two triglyceride substrates with different chain length: tributyrin ($C_{4:0}$) and triolein ($C_{18:1}$). The tests were done at pH 9 by the LU and SLU methods described above. The following variants were found to have ratio of triolein activity to tributyrin activity higher than the parent enzyme (Lipolase):

E1SPIRPRP +G91A +D96N +E99K +Q249R
E1SPCRPRP+ S83T+ N94K+ D96L+ E239C+ Q249R
G266D
E1SPIRPRP +D62A +E99K +Q249R
E1SPIRPRP +D62G +E99K +Q249R
E1SPIRPRP +D62V +E99K +Q249R
E1SPIRPRP +R84W +E99K +Q249R
E1SPIRPRP +R84K +E99K +Q249R
E1SPIRPRP + K98D +E99K +Q249R
E1SPIRPRP + E99K +Q249R + 270PGLPFKRV
E1SPPCGRRP + E99N +N101S +T231K +R232G +D234G +E239C +Q249R
E1SPIRPRP +E99K +Q249R + 270PWPARLGRL
L93K +D96G
G91A +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A+ 271G+ 272G+ 273F (+274S)
E1SPCRPRP +V60G +E99N +S119G +R209P +E239C +Q249R
G266A
G266E
G266V
G263Q +L264A +I265T +G266D +T267A
G266L
G263A +G266A
E1SPCRPRP +E239C +Q249R +G266A
E1SPCRPRP +E239C +Q249R +G266S
D96S + G266A
D96S + G266S

D96S + G266W
L264I +I265N +G266T +T267GL
E1SPPCGRRP +D96S +E239C +Q249R +L264I +I265N +G266T +T267GL
D96F +G266A
D96F +G266S
E1SPPCGRRP +E99N +E239C +Q249R +G266A
E1SPPCGRRP + D96S +E239C +Q249R +G266A
E1SPPCGRRP + D96S +E239C +Q249R +G266S
D62A + S83T
E1SPPCGRRP +K98D +E99N +E239C +Q249R
T231R +N233R +270CP
E1SPPCGRRP +E99N +E239C +Q249R +270MD
E1SPPCGRRP + D62A +S83T +E99N +E239C +Q249R
D62A +S83T + G91A +E99K +T231R +N233R +Q249R
V60G +D62A +S83T +R84K +D96W +G266D
L259N
L259R
L259M
L259Q

Example 10: DGDGase activity

The hydrolytic activity towards DGDG (di-galactosyl-di-glyceride) was determined by monolayer assay (DGDGase activity) as follows:

On a thoroughly purified surface of a buffer solution (10 mM NaOAc, pH 5.5; 1 mM CaCl2, 25°C; 10 mM beta-cyclodextrin (Sigma C-4767)) a monolayer of DGDG (Sigma (D4651)) is spread from a chloroform solution. After relaxation of the monolayer (evaporation of chlorofom) the surface pressure is adjusted to 15 mN/m. A solution containing approximately 60 µg (micro gram) enzyme is injected through the monolayer into the subphase of the re-action compartment (cylinder with surface area 2230 mm^2 and reaction volume 56570 mm^3) in the "zero-order trough". Enzymatic activity is manifested through increased speed of a mobile barrier compressing the monolayer in order to maintain constant surface pressure as insoluble substrate molecules are hydrolysed into more water soluble reaction products (in presence of beta cyclodextrin).

The result is considered positive for DGDGase if the barrier moves at more than 1 mm/min. A number of variants of Lipolase were tested and found to have DGDGase activity, whereas the parent enzyme (Lipolase) did not have this activity. Modifications in parentheses were uncertain.

Example 11: Thermostability of variants

The thermostability of variants was tested by DSC (Differential Scanning Calorimetry) by heating at 90 deg/hr at pH 5 (50 mM acetate buffer), pH 9 (50 mM borate buffer) or pH 10 (50 mM glycine buffer). Variants NO. 1, 2, 3, 5 and 8 listed in Example 5 were tested, and the denaturation peak (T_d) in each case was found to be in the range 54-69°C.

Example 12: Degumming of vegetable oil

Rape seed oil was treated with two variants of the invention, essentially as described in Example 6 of WO 98/18912 (Novo Nordisk).

One variant of Lipolase was tested at an enzyme dosage of 0.6 mg of enzyme protein per kg of oil. Results of tests at various pH and temperatures showed optimum performance at pH 5.7, 35-45°C, where a final P content of 4 ppm was reached. A separate experiment at 45°C, pH 6 showed that a final P content of 4 ppm could be reached at an enzyme dosage as low as 0.15 mg/kg.

A similar experiment with another Lipolase variant showed optimum performance at 40°C, pH 5.0-5.5. The enzyme dosage was 0.3 mg/kg.

A degumming experiment was made with a third Lipolase variant, using rape seed oil at 45°C, pH 5, 1.8 mg enzyme/kg oil. For comparison, a similar experiment was made with the parent lipase (Lipolase) at 18 mg/kg. The results showed that good degumming (<10 ppm residual P content) was obtained in 3.4 hours with the variant.

The parent lipase (Lipolase) was found to have very little degumming effect, even at 10 times higher enzyme dosage.

Example 13: Baking tests

A variant of Lipolase was evaluated in baking tests as follows.

Doughs were prepared from Meneba flour according to the European straight dough method (ABF-SP-1201.01) with 40 ppm of ascorbic acid. Various combinations of additives at the following dosages were used: the lipase variant at 0, 0.25, 0.5 or 1.5 mg/kg; phospholipid (lecithin) at 0 or 10 g/kg; and endo-amylase at 0 or 750 MANU/kg.

The endo-amylase was maltogenic amylase from *B. stearothermophilus* (tradename Novamyl ®). One MANU (Maltogenic Amylase Novo Unit) is defined as the

amount of enzyme required to release one µmol of maltose per minute at a concentration of 10 mg of maltotriose substrate per ml of 0.1 M citrate buffer, pH 5.0 at 37 °C for 30 minutes.

After baking, the loaves were cooled, and the loaf volume, crumb firmness and softness were evaluated after approximately 2 hours. The evaluation was repeated after 1, 3 and 7 days storage at 22°C wrapped in double plastic bags.

Firmness of crumb was measured using a texture analyzer TA-XT2 from Stable Micro Systems (probe diameter 40 mm).

Softness in gram was measured as the force needed to press a probe 6,25 mm into a crumb of a 25 mm thick slice of bread (25 % penetration).

The results showed that the addition of 1.5 mg of the variant increased the loaf volume. The results for firmness and elasticity show that the variant gives significantly softer crumb and significantly better elasticity from day 0 to day 7.

Example 14: Off-odor development from lipases with varying chain-length specificity

The development of off-odor from lipases with different chain-length specificity was evaluated in whole milk. The developed butyric acid/sour odor was evaluated by sniffing the samples after heating.

25 ml whole milk was placed in 100 ml blue cap flasks (with caps) in a 32°C water bath. Of each of the lipases listed below, 0.2 mg enzyme protein per litre milk was added to the flasks. The temperature was raised to 45°C, and evaluation took place after 15 and 105 minutes.

The lipases tested were Lipolase and variants thereof. For each lipase, the chain-length specificity is expressed as the ratio of activities on triolein (SLU) and tributyrin (LU).

Three persons evaluated the samples and agreed on the ranking shown below

+ Detectable smell

++ Clear and characteristic butyric acid and/or sour odor

+++ Strong butyric acid and/or sour odor

Three variants of Lipolase having a higher SLU/LU ratio than Lipolase were found to have less malodor than the parent lipase (Lipolase).

Example 15: Test of lipase related malodour on textile

Soiling:

Cotton textile was soiled with a dairy product as described here. 50 mg of butter was applied over an area of approximately 30 cm^2 in an even spot. The soiled t x-til was aged for 24 hours at ambient conditions.

Washing procedure:

Washing of the soiled textile was done in a Terg-O-tomet r using a commercial detergent(5 g/l) with and without lipase(1250 and 5000 LU/l). The washing was done at 30°C for 20 min at 100 rpm. After washing the swatches were left overnight to dry at ambient conditions.

Sensory analysis:

Next day, the malodour was assessed by a sensory panel consisting of at least 10 trained assessors. Samples were kept in tight glass jars and left at least 30 minutes between every evaluation for accumulation of . Swatches were taken out and the malodour assessed on the textile. The butyric acid malodour was scored according to the scale below. As a reference the sample washed without lipase was used.

0. Fainter smell than reference
1. Same as reference
2. Slightly stronger than reference
3. Definitely stronger than reference
4. Stronger than 3.

Variants of Lipolase with an increased ratio of triolein/tributyrin activities (increased SLU/LU ratio) were found to give a fainter smell from butter stains than the parent enzyme (Lipolase). A separate washing experiment showed that the variants, like the parent enzyme, were effective in the removal of lard stains.

Alternative methods

The intensity of butyric acid from dairy stains on fabric can also be evaluated by instrumental analysis:

1. By Head Space Gas Chromatography, or
2. By extraction of the odours from fabric followed by Gas Chromatography

CLAIMS

1. A method of producing a lipolytic enzyme variant comprising:

a) selecting a substrate and an ester bond of interest,

b) selecting a parent lipolytic enzyme having an alcohol binding site having a glycerol part with an sn2 position,

c) selecting one or more amino acid residues in the parent lipolytic enzyme at positions which in a three-dimensional structure of the parent lipolytic enzyme and a substrate is(are) within 10 Å of the C atom at the sn2 position of the glycerol part of a substrate triglyceride,

d) making alterations each of which is an insertion, a deletion or a substitution of the amino acid residue,

e) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b),

f) preparing the variant resulting from steps b-d,

g) testing the activity of the variant on the ester bond in the substrate, and

h) selecting a variant having an altered activity on the ester bond.

2. A method of producing a lipolytic enzyme variant comprising:

a) selecting a substrate and an ester bond of interest,

b) selecting a parent lipolytic enzyme having a structure comprising a catalytic triad consisting of an active Ser, an active Asp and an active His residue,

c) selecting one or more amino acid residues in the parent lipolytic enzyme belonging to a set E defined by the following steps:

i) aligning the structure of the lipolytic enzyme with *Rhizomucor miehei* lipase structure 4TGL comprising a catalytic triad and an inhibitor phosphorus atom (4TGL-inhP), so as to minimize the sum of squares of deviation between atoms of the catalytic triads of the two structures,

ii) defining a set A consisting of atoms of the lipolytic enzyme inside a sphere of radius 18 Å with center at 4TGL-inhP,

iii) forming a first plane defined by 4TGL-inhP, the Cα atom of the active Ser residue of the parent lipolytic enzyme, and the Cα atom of th active Asp residue of the parent lipolytic enzyme and defining a set B as a subset of set A consisting of atoms on the same side of the first plane as the Cα atom of the active His residue of the parent lipolytic enzyme,

iv) forming a second plane defined by 4TGL-inhP, the Cα atom of the active Ser residue of the parent lipolytic enzyme, and the Cα atom of the active His residue of the parent lipolytic enzyme and defining a set C as a subset of set A consisting of atoms on the opposite side of the second plane from the Cα atom of the active Asp residue of the parent lipolytic enzyme,

v) forming a set D consisting of atoms belonging to the union of sets B and C, and having a solvent accessibility of 15 or higher, and

vi) forming set E consisting of amino acid residues in the structure which comprise an atom belonging to set D or an atom belonging to the union of sets B and C and located less than 3.5 Å from an atom belonging to set D,

d) making alterations each of which is an insertion, a deletion or a substitution of the selected amino acid residues,

e) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than d),

f) preparing the variant resulting from steps d) - f), and

g) screening for a variant having an altered activity on the selected ester bond.

3. A method of producing a lipolytic enzyme variant comprising:

a) selecting a substrate and an ester bond of interest,

b) selecting a parent lipolytic enzyme having an active site comprising an active His residue,

c) selecting one or more amino acid residues in the amino acid sequence of the parent lipolytic enzyme between the active His residue and the C-terminal,

d) making alterations each of which is an insertion, a deletion or a substitution of the amino acid residue,

e) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b),

f) preparing the variant resulting from steps b-d,

g) testing the activity of the variant on the ester bond in the substrate, and

h) selecting a variant having an altered activity on the ester bond.

4. A method of producing a lipolytic enzyme variant comprising:

a) selecting a substrate and an ester bond of interest,

b) selecting a parent lipolytic enzyme,

c) selecting one or more among 10 amino acid residues at the C-terminal,

d) making alterations each of which is an insertion, a deletion or a substitution of the selected amino acid residues,

e) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than c),

f) preparing the variant resulting from steps c) - e), and

g) screening for a variant having an altered activity on the selected ester bond.

5. A method of producing a lipolytic enzyme variant comprising:

a) selecting a substrate and an ester bond of interest,

b) selecting a parent lipolytic enzyme having a lid,

c) selecting one or more amino acid residues in the lid,

d) making alterations each of which is an insertion, a deletion or a substitution of the selected amino acid residues,

e) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than c),

f) preparing the variant resulting from steps c) - e), and

g) screening for a variant having an altered activity on the selected ester bond.

6. The method of any preceding claim wherein the lipolytic enzyme is native to an eukaryote, preferably to a fungus, and most preferably belongs to the *Humicola* family or the *Zygomycete* family.

7. A method of producing a lipolytic enzyme variant comprising:

a) selecting a substrate and an ester bond of interest,

b) selecting a parent lipolytic enzyme from the *Humicola* family,

c) selecting one or more amino acid residues corresponding to any of amino acids 20-25, 56-64, 81-85 and 255-269 in the *Humicola lanuginosa* lipase

d) making alterations each of which is an insertion, a deletion or a substitution of the amino acid residue,

e) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than c),

f) preparing the variant resulting from steps b-e,

g) testing the activity of the variant on the ester bond in the substrate, and

h) selecting a variant having an altered activity on the ester bond.

8. The method of the preceding claim wherein the parent lipolytic enzyme is the lipase of *Humicola lanuginosa* strain DSM 4109.

9. The method of any preceding claim wherein the altered activity is a higher activity towards a C_4-C_8 triglyceride or a higher ratio between the activity towards a C_{16}-C_{20} acyl bond in a triglyceride and the activity towards a C_4-C_8 acyl bond in a triglyceride.

10. The method of the preceding claim wherein the parent lipolytic enzyme belongs to the *Humicola* family, preferably the lipase of *Humicola lanuginosa* strain DSM 4109, and the selected amino acid residues comprise an amino acid corresponding to Y21, E56, D57, V60, G61, D62, R81, S83, R84, L259, Y261 or G266 in the *Humicola lanuginosa* lipase.

11. The method of any of claims 1-8 wherein the altered activity is a higher phospholipase activity.

12. The method of the preceding claim wherein the parent lipolytic enzyme has a phospholipase activity below 50 PHLU/mg and/or a ratio of phospholipase activity to lipase activity below 0,1 PHLU/LU.

13. The method of claim 11 or 12 wherein the parent lipolytic enzyme belongs to the *Humicola* family, preferably the lipase of *Humicola lanuginosa* strain DSM 4109, and the selected amino acid residues comprise an amino acid corresponding to R81, R84, S85, or 263-267 (e.g. G266 or T267) in the *Humicola lanuginosa* lipase.

14. The method of any of claims 11-13 wherein the alterations comprise insertion of a peptide extension at the C-terminal, preferably comprising 1-5 amino acid residues, the first preferably being A, P or D, the second (if present) preferably being V, G or R, the third (if present) preferably being V, G or R, the fourth (if present) preferably being F, and the fifth (if present) preferably being S.

15. The method of any of claims 1-8 wherein the altered activity is a higher hydrolytic activity on a digalactosyl-diglyceride.

16. The method of the preceding claim wherein the parent lipolytic enzyme belongs to the *Humicola* family, preferably the lipase of *Humicola lanuginosa* strain DSM 4109, and the selected amino acid residues comprise an amino acid corresponding to 21, 23, 26, 57, 62, 81, 83, 84, 85, 266, 267 or 269 in the *Humicola lanuginosa* lipase.

17. A lipolytic enzyme which is a variant of a parent lipolytic enzyme having an alcohol binding site having a glycerol part with an sn2 position, which variant:

a) comprises an alteration which is an insertion, a deletion or a substitution of an amino acid residue, at a position which in a three-dimensional structure of the parent lipolytic enzyme and a substrate is within 10 Å of the C atom at the sn2 position of the glycerol part of a substrate triglyceride, and

b) has an altered activity on an ester bond in the substrate.

18. A lipolytic enzyme which is a variant of a parent lipolytic enzyme having a lid, which variant:

a) comprises an alteration which is an insertion, a deletion or a substitution of an amino acid residue in the lid,

b) has an altered activity on an ester bond in the substrate.

19. A lipolytic enzyme which is a variant of a parent lipolytic enzyme, which variant:

a) comprises an alteration which is an insertion, a deletion or a substitution of one or more within 10 amino acid residues of the C-terminal,

b) has an altered activity on an ester bond in a substrate.

20. The lipolytic enzyme of any of claims 17-19 wherein the parent lipolytic enzyme is native to an eukaryote, preferably to a fungus, and most preferably belongs to the *Zygomycete* family.

21. The lipolytic enzyme of the preceding claim wherein the parent lipolytic enzyme belongs to the *Humicola* family, preferably the lipase of *Humicola lanuginosa* strain DSM 4109.

22. A lipolytic enzyme which:

a) is a polypeptide having an amino acid sequence which has at least 90 % homology with a reference lipolytic enzyme of the *Humicola* or *Zygomycete* family;

b) compared to said wild-type lipase comprises an amino acid modification which is:

i) a substitution, deletion or insertion at a position corresponding to A20, Y21, G23, K24, N25, V63, R81, G82, R84, A257, W260, Y261, F262 or G266 in the *Humicola lanuginosa* DSM 4109 lipase;

ii) a substitution of an amino acid corresponding to C268 or L269 in said lipase;

iii) a substitution corresponding to V60G, D62E, L93K, L97Q, K98E,F, E99D, P256A, G263E,Q,R,F,N, L264A,C,P,F,G,V,I, I265L,N,F or T267A,Q,P,S,V,E in said lipase;

iv) an insertion corresponding to T267GS or T267GL in said lipase;

v) a peptide extension at the C-terminal which is A, P, MD, CP, AG, DG, AGG, PVGF, AGRF, PRGF, AGGF or AGGFS;

vi) a peptide extension at the C-terminal of 40-50 amino acids; or

vii) a truncation of 1, 2, 3, 4, 5 or 6 amino acids at the C-terminal; and

c) has an altered activity on an ester bond in a substrate compared with the lipolytic enzyme of the *Humicola* family.

23. The lipolytic enzyme of the preceding claim wherein the amino acid modification corresponds to R84K,L,W, W260H,Q,C, G266A,C,D,N,L,I,S,T,P,V,F,W,E,K,R,Y or L269N,I,S.

24. The lipolytic enzyme of claim 22 or 23 which further comprises one or more further amino acid modification which is a substitution, a deletion or an insertion corresponding to any of positions 22, 56-59, 61, 64, 83, 85, 91, 94, 249, 255 or 259 preferably S83T, G91A, N94D, D96S,W,F,G, Q249R or L259N,R,S,M,Q.

25. The lipolytic enzyme of any of claims 22-24 which further comprises a substitution corresponding to D62A,G,V, K98D, E99K, P256T, G263A and/or I265T,G,V.

26. The lipolytic enzyme of any of claims 22-25 which comprises a peptide extension at the N-terminal.

27. The lipolytic enzyme of any of claims 22-26 wherein the reference lipolytic enzyme is the lipase from *Humicola lanuginosa*.

28. The lipolytic enzyme of any of claims 22-26 wherein the reference lipolytic enzyme is the lipase from *Rhizomucor miehei*.

29. A lipolytic enzyme which:

a) is a polypeptide having an amino acid sequence which has at least 90 % homology with a reference enzyme which is the lysophospholipase from *Aspergillus foetidus*, the ferulic acid esterase from *Aspergillus niger*, the ferulic acid

esterase from *Aspergillus tubigensis* or phospholipase A1 from *Aspergillus oryzae*,

b) compared to said reference enzyme comprises an amino acid alteration which is a substitution, deletion or insertion at a position corresponding to 20-25, 56-64, 81-85, 91-98, 255-257 or 259-269 in the *Humicola lanuginosa* lipase, and

c) has an altered activity on an ester bond in a substrate compared with the reference enzyme.

30. The lipolytic enzyme of claim 29 wherein the alteration is at a position corresponding to Y21, E56, D57, V60, G61, D62, S83, R84, G91, L93, N94, D96, L97, K98, E99, P256, W260, Y261, G263, L264, I265, G266, T267 or L269 in the *Humicola lanuginosa* lipase

31. The lipolytic enzyme of claim 29 or 30 wherein the alteration is an extension or a truncation at the C-terminal, preferably by 1-5 amino acids.

32. The lipolytic enzyme of any of claims 22-31 wherein the altered activity is a higher ratio between the activity towards a C_{16}-C_{20} acyl bond in a triglyceride and the activity towards a C_4-C_8 acyl bond in a triglyceride or a higher activity towards a C_4-C_8 triglyceride.

33. The lipolytic enzyme of claim 32 which comprises an amino acid modification at a position corresponding to Y21, E56, D57, V60, G61, D62, R81, S83, R84, L259, Y261 or G266 in the *Humicola lanuginosa* lipase.

34. The lipolytic enzyme of any of claims 22-31 wherein the altered activity is a higher phospholipase activity.

35. The lipolytic enzyme of the preceding claim which comprises an amino acid modification at a position corresponding to R81, R84, S85, G263, L264, I265, G266, T267 or L269 in the *Humicola lanuginosa* lipase, preferably a substitution corresponding to G263A,E,Q,R; L264A,C,P,Q; I265L,N,T; G266A, C, D, N, L, I, S, T, P, V or T267A,Q or L269N.

36. The lipolytic enzyme of claims 34 or 35 which has a phospholipase activity greater than 0.1 nmol/min in a monolayer assay at pH 5 as described herein and/or a phospholipase activity greater than 100 PHLU/mg (preferably greater than 500 PHLU/mg) and/or a ratio of phospholipase activity to lipase activity greater than 0.1 PHLU/LU (preferably greater than 0.5 PHLU/LU).

37. The lipolytic enzyme of any of claims 34-36 wherein the parent lipolytic enzyme has a phospholipase activity below 50 PHLU/mg and/or a ratio of phospholipase activity to lipase activity below 0,1 PHLU/LU.

38. The lipolytic enzyme of any of claims 34-37 which comprises a peptide extension at the C-terminal, preferably comprising 1-5 amino acid residues, the first preferably being A, P or D, the second (if present) preferably being V, G or R, the third (if present) preferably being V, G or R, the fourth (if present) preferably being F, and the fifth (if present) preferably being S.

39. The lipolytic enzyme of any of claims 34-38 which comprises a deletion of amino acid residues at positions corresponding to positions C268 and L269 in the lipase of *Humicola lanuginosa* strain DSM 4109.

40. The lipolytic enzyme of any of claims 22-31 wherein the altered activity is a higher hydrolytic activity on a digalactosyl-diglyceride.

41. The lipolytic enzyme of the preceding claim which comprises an amino acid modification at a position corresponding to Y21, G23, N26, D57, D62, R81, S83, R84, S85, G266, T267 or L269 in the *Humicola lanuginosa* lipase, preferably comprising two or more such alterations, most preferably further comprising one or more alterations in the lid region

42. The lipolytic enzyme of any of claims 22-41 which comprises an alteration in the lid which is a substitution of a negatively charged amino acid residue with a neutral or positively charged amino acid residue, or a substitution of a neutral amino acid residu with a positively charged amino residue.

43. The lipolytic enzyme of the preceding claim which comprises an alteration in the lid at a position corresponding to position G91, D96 and/or E99 in the *Humicola lanuginosa* lipase, preferably a substitution which is G91A, D96S,W,F or E99K.

44. A lipolytic enzyme which is a variant of a parent lipase derived from *Humicola lanuginosa* strain DSM 4109 comprising the modifications E1E,D,A+ G91G,A,S,T+ N94N,D+ D96D,G,F,W+ E99E,K+ G225G,R,K+ G263Q,N+ L264L,A,V+ I265I,T,S+ G266G,A,V,S,D,E+ T267T,A,V+ L269L,I,N,Q.

45. The lipolytic enzyme of the preceding claim which further comprises SPIRR as a peptide extension at the N-terminal and/or AGGF or AGGFS as a peptide extension at the C-terminal.

46. A DNA sequence encoding the lipolytic enzyme of any of claims 22-45.

47. A vector comprising the DNA sequence of the preceding claim.

48. A transformed host cell harboring the DNA sequence of claim 46 or the vector of claim 47.

49. A method of producing the lipolytic enzyme of any of claims 22-45 comprising

a) cultivating the cell of claim 48 so as to express and preferably secrete the lipolytic enzyme, and

b) recovering the lipolytic enzyme.

50. A process for preparing a dough or a baked product prepared from the dough which comprises adding the lipolytic enzyme of any of claims 22-45 to the dough, wherein the lipolytic enzyme preferably has phospholipase or digalactosyl diglyceride activity.

51. The process of the preceding claim which further comprises adding to the dough an endo-amylase and/or a phospholipid.

52. The process of claim 50 or 51 wherein the endo-amylase is from *Bacillus*, and is preferably a maltogenic amylase from *B. stearothermophilus*,

53. A process for reducing the content of phospholipid in an edible oil, comprising treating the oil with the lipolytic enzyme of any of claims 34-39, 44 or 45 so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil.

54. A process for improving the filterability of an aqueous solution or slurry of carbohydrate origin which contains phospholipid, which process comprises treating the solution or slurry with the lipolytic enzyme of any of claims 34-39, 44 or 45, wherein the solution or slurry preferably contains a starch hydrolysate, particularly a wheat starch hydrolysate.

55. A detergent composition comprising a surfactant and the lipolytic enzyme of any of claims 22-45.

Primers

4244 (SEQ ID NO: 1): 5'-TCA AGA ATA GTT CAA ACA AGA AGA-3'

AP (SEQ ID NO: 2): 5'-GGT TGT CTA ACT CCT TCC TTT TCG-3'

FOL14 (SEQ ID NO: 3): 5'-TGT CCC YMG WCT CCC KCC K-3'

FOL15 (SEQ ID NO: 4): 5'-GAA GTA MYR YAG RTG MGC AGS RAT ATC-3'

FOL16 (SEQ ID NO: 5): 5'-GAT ATY SCT GCK CAY CTR YRK TAC TTC-3'

H7 (SEQ ID NO: 6): 5'-CGG AAT GTT AGG CTG GTT ATT GC-3'

KBoj 14 (SEQ ID NO: 7): 5'-CTT TTC GGT TAG AGC GGA TG-3'

KBoj 32 (SEQ ID NO: 8): GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CTA 122 345 345 S67 C8A 91011 S1213 1.14.15 161718 T1920 C2122 GAA GTA CCA TAG GTG CGC AG23 GAT ATC CGG

KBoj 33: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CGC CGC GCA CTA C8A 91011 S1213 1.14.15 161718 T1920 C2122 GAA GTA CCA TAG GTG CGC AG23 GAT ATC CGG

KBoj34: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CTA 122 345 345 S67 201818 91011 S1213 1.14.15 161718 T1920 C2122 GAA GTA CCA TAG GTG CGC AG23 GAT ATC CGG

KBoj36: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CTA GTT ACA GGC GTC AGT CGC CTG GAA G

KBoj37: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CTA AGC GTT ACA GGC GTC AGT CGC CTG G

KBoj38: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CTA ACC AGC GTT ACA GGC GTC AGT CGC C

KBoj39: GTA AGC GTG ACA TAA CTA ATT ACA TCA TGC GGC CCT CTA GAG TCG ACC CAG CCG CTA GCC ACC AGC GTT ACA GGC GTC AGT C

Distribution of nucleotides for each doped position

1: A 90, C 10

2: G 3,A 91,T 3,C 3

3: A 25, T 75

4: G 2, A 4, T 5, C 89

5: G 2, A 13, T 4, C 81

6: G 91, A 3, T 3, C 3

7: G 48, A 2, T 2, C 48

8: A 92, T8

9: A 97, T 3

10: G 1, A 1, T 1, C 97

11: G 1, A 97, T 1, C 1
12: G 94, A 2, T 2, C 2
13: G 1, A 1, T 91, C 7
14: G 1, A 1, T 7, C 91
15: G 2, A 2, T 2, C 94
16: A 80, T 20
17: G 6, A 90, T 2, C 2
18: G 2, A 2, T 94, C 2
19: G 5, A 91, T 4
20: G 96, C4
21: G 4, T 5, C 91
22: G 4, C 96
23: G 94, C 3, T 3

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          1                                                  50
  rhimi   SIDGGIRAAT SQEINELTYY TTLSANSYCR TVIPGA...T WDC..IHCDA
  rhidl   SDGGKVVAAT TAQIQEFTKY AGIAATAYCR SVVPGN...K WDC..VQCQK
  SP400   --------EVS QDLFNQFNLF AQYSAAAYCG KNNDAPAGTN ITCTGNACPE
    Pcl   --------DVS TSELDQFEFW VQYAAASYYE ADYTAQVGDK LSCSKGNCPE
FoLnp11   --------GVT TTDFSNFKFY IQHGAAAYC. .NSEAAAGSK ITCSNNGCPT

          51                                                 100
  rhimi   TE..DLKIIK TWS.TLIYDT NAMVARGDSE KTIYIVFRGS SSIRNWIADL
  rhidl   WV.PDGKIIT TFT.SLLSDT NGYVLRSDKQ KTIYLVFRGT NSFRSAITDI
  SP400   VEKADATFLY SFEDSGVGDV TGFLALDNTN KLIVLSFRGS RSIENWIGNL
    Pcl   VEATGATVSY DFSDSTITDT AGYIAVDHTN SAVVLAFRGS YSVRNWVADA
FoLnp11   VQGNGATIVT SFVG.SKTGI GGYVATDSAR KEIVVSFRGS INIRNWLTNL

          101                                                150
  rhimi   TFVPVSY.PP VSGTKVHKGF LDSYGEVQNE LVATVLDQFK QYPSYKVAVT
  rhidl   VFNFSDY.KP VKGAKVHAGF LSSYEQVVND YFPVVQEQLT AHPTYKVIVT
  SP400   NFDLKEINDI CSGCRGHDGF TSSWRSVADT LRQKVEDAVR EHPDYRVVFT
    Pcl   TFVHTNP.GL CDGCLAELGF WSSWKLVRDD IIKELKEVVA QNPNYELVVV
FoLnp11   DFGQEDC.SL VSGCGVHSGF QRAWNEISSQ ATAAVASARK ANPSFNVIST

          151                                                200
  rhimi   GHSLGGATAL LCALDLYQRE EGLSSSNLFL YTQGQPRVGD PAFANYVVST
  rhidl   GHSLGGAQAL LAGMDLYQRE PRLSPKNLSI FTVGGPRVGN PTFAYYVEST
  SP400   GHSLGGALAT VAGADLRGN. .GY...DIDV FSYGAPRVGN RAFAEFLTVQ
    Pcl   GHSLGAAVAT LAATDLRGK. .GYP..SAKL YAYASPRVGN AALAKYITAQ
FoLnp11   GHSLGGAVAV LAAANLRVG. .GT...PVDI YTYGSPRVGN AQLSAFVSNQ

          201                                                250
  rhimi   G.IPYRRTVN ERDIVPHLPP AAFGFLHAGE EYWITD.NSP ......ETVQ
  rhidl   G.IPFQRTVH KRDIVPHVPP QSFGFLHPGV ESWIKS.GT. ......SNVQ
  SP400   TGGTLYRITH TNDIVPRLPP REFGYSHSSP EYWIKS.GTL V.PVTRNDIV
    Pcl   G..NNFRFTH TNDPVPKLPL LSMGYVHVSP EYWITS.PNN A.TVSTSDIK
FoLnp11   A.GGEYRVTH ADDPVPRLPP LIFGYRHTTP EFWLSGGGGD KVDYTISDVK

          251                                                300
  rhimi   VCTSDLETSD CSNSIVPFT. .SVLDHLSYF GINTGLCT-- ----------
  rhidl   ICTSEIETKD CSNSIVPFT. .SILDHLSYF DINEGSCL-- ----------
  SP400   KIEGID.ATG GNNQP.NIP. .DIPAHLWYF .GLIGTCL-- ----------
    Pcl   VIDGDV.SFD GNTGTGLPLL TDFEAHIWYF .VQVDAGKGP GLPFKRV---
FoLnp11   VCEGAA.NLG CNGGT.LGL. .DIAAHLHYF .QATDACNAG GFSWRRYRSA

          301                                          338
  rhimi   ---------- ---------- ---------- -----...
  rhidl   ---------- ---------- ---------- -----...
  SP400   ---------- ---------- ---------- -----...
    Pcl   ---------- ---------- ---------- -----...
FoLnp11   ESVDKRATMT DAELEKKLNS YVQMDKEYVK NNQARS..
```

Fig. 1

Alignment of lipase sequences